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A STUDY OF THE CELL MEMBRANE AND ENZYMES  
OF HALOBACTERIUM SALINARIUM

by Francis Bellingham



A thesis submitted in partial fulfilment of the  
requirements for the award of the degree of Doctor  
of Philosophy of the University of Warwick.

[1972]



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### Preface

The work described in this thesis was carried out in the School of Molecular Sciences of the University of Warwick during the period October 1968 to September 1970.

I wish to thank Professor V. M. Clark for providing the facilities necessary for the research and Drs J. Stevenson and D. E. Griffiths for their guidance and encouragement.

I would also like to thank the Scientific Research Council for the award of a Research Studentship.

A Man would do nothing, if he waited  
until he could do it so well that no-one  
would find fault with what he had done.

Cardinal Newman (1801-1890)

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### Summary

1. A colourless mutant, strain 1M, of the extreme halophilic Halobacterium salinarium strain 1 was shown to be more sensitive to changes of NaCl concentration than the wild-type. The levels and cellular distribution of several enzymes were determined in each strain and the sensitivity to changes of NaCl investigated for selected membrane bound and soluble enzymes. ATPase, acid phosphatase, alkaline phosphatase and glycerol dehydrogenase of both strains all showed typical halophilic enzyme responses with optimum activity only in the presence of 2.5 - 4.0M salt.
2. Malate dehydrogenase from strain 1 was extracted and purified. The purified preparation showed an 850 fold increase of specific activity over the crude extract. The fluorescence probe 1-8 ANS was used to investigate possible conformational changes induced in the purified enzyme by variations of NaCl concentration. Results indicate that upon removal of salt ANS fluorescence is enhanced possibly implying that unfolding of the protein occurs.
3. The cell membrane of strain 1 and 1M has been prepared from cell envelopes and purified by gel-filtration on Agarose A50m. Purification resulted in the release of a low M.W. ( $\sim 2500$ ) polar protein which contained some nucleotide material. Electron micrographs of crude and purified membrane showed abundant triple-



layered structures and vesicles typical of classical unit membrane structures. Urea caused the release of highly polar fractions which were isolated by PGE and characterised by gel-filtration and amino acid analysis.

4. The membrane lipids of strain 1 and 1M were extracted and a preliminary characterisation carried out. Besides the lack of carotenoid in strain 1M other differences in the lipid composition were found between the two strains. Strain 1M contained less menaquinone and at least two different phospho-lipids. Lipid-free membrane protein was extracted by a method combining organic solvent extraction and gel-filtration. The membrane protein of both strains was shown to be apolar in character and heterogenous by separation on PGE. A structural protein fraction which may be similar to SP obtained from other membrane sources was isolated from purified membrane.

5. Purified membrane was solubilised with mixed anionic detergents (sodium dodecyl sulphate, sodium cholate and sodium deoxycholate) and separated by gel-filtration into protein-rich and lipid-rich fractions. The lipids from each of these fractions were extracted and compared with total membrane extracts. The lipid component of the protein-rich fraction appeared to contain mainly polar lipids.

6. The protein-rich and lipid-rich fractions were reaggregated in the presence of  $10\text{mM Mg}^{2+}$  or  $5\text{mM}$  spermine to form reconstituted

membrane. Reconstituted membrane appeared in e.m.s. to contain unit membrane structures. Like native membrane, reconstituted membrane could only be disrupted by anionic detergents indicating that hydrophobic bonding was of prime importance in the interaction between protein and lipid. Experiments in which membrane protein was labelled with the fluorescent probe DNS indicated that the membrane protein and lipid fractions were stabilised to some extent when they interacted. However, significant differences were observed in the behaviour of dansylated native membrane and dansylated reassembled membrane indicating that the organisation of protein and lipid in each may not be directly comparable.

## Chapter I: Introduction

Halophilic bacteria have a specific requirement for sodium chloride. This phenomenon is referred to as halophilism and has been reviewed extensively by Flannery (1956), Ingram (1957) and Larsen (1962), (1967). Marine bacteria require 2-5% NaCl as an essential constituent of their growth medium. Other bacteria, however, may tolerate or even require higher levels. Moderate halophiles grow optimally in media containing 5-20% NaCl, whilst extreme halophiles require a minimum level of 10-15% NaCl and grow best at concentrations between 20% and saturated NaCl (approximately 30%). The principal organism used in the work described in this thesis was Halobacterium salinarium, an example of an extreme halophile.

Distribution and growth: The main genus of the extreme halophiles is Halobacterium. "Bergey's manual" (1957) lists five species, H. salinarium, H. cutirubrum, H. halobium, H. morismortii and H. trapanicum. They occur naturally in habitats containing high levels of NaCl. For example saline soils and concentrated brines such as occur in the localities of the Dead Sea and Great Salt Lake, U.S.A. or evaporation pans of salt works. Mined salt is usually not contaminated but marine solar salt often contains large numbers of extreme halophiles (Larsen, 1962) which may remain viable under the conditions of extreme dehydration associated with salt crystals, even though they do not form spores.

For this reason, solar salt used as a preservative has frequently caused extensive microbial spoilage, e.g. "pinkeye" of salted fish (Morrison and Kennedy, 1922 and Shewan, 1942) and "red-heat" of salted hides (Lochead, 1934).

The Halobacteria are Gram-negative rods, obligate aerobes and if motile lophotrichously flagellated. H. salinarium strain 1 is non-vacuolated and motile. Cultures are red and give off an unpleasant odour. Since extreme halophiles have rather unusual nutritional requirements, proteins or amino acids being the preferred carbon sources, the odour from cultures may be due to the presence of nitrogen containing degradation products like putrescine. Carbohydrates are usually not metabolised. Culture media normally contain protein digests (e.g. peptone, yeast autolysate), suitably high levels of NaCl and comparatively low concentrations of  $K^+$  and  $Mg^{2+}$ , (Brown and Gibbons, 1955). Trace element requirements are usually satisfied if media are prepared in tap water (Sehgal and Gibbons, 1960). Defined media tend to be fairly complex including at least 10 amino acids, nucleotides, and glycerol (Dundas et al, 1963 and Onishi et al, 1965).  $Na^+$  can be partially replaced by  $K^+$  and to a lesser extent by  $Mg^{2+}$ , (Brown and Gibbons, 1955; Christian, 1956; Weber, 1949), but if the  $Na^+$  level is cut by too much normal growth is not maintained.

The cell envelope of Halobacteria: Exposure of Halobacteria to dilute salt solutions leads to a lysis of the cells. When the

dilution is carried out by step-wise changes of NaCl concentration the rod shaped cells change their shape via irregular forms to spheres. At 5-10% NaCl the spheres suddenly lyse. Mohr and Larsen (1963) have shown that the transformation from rods to spheres occurs without a change of total cell volume implying that osmotic equilibrium is maintained throughout the dilution procedure. These workers also demonstrated that certain ions which interact weakly with proteins tend to protect the rod-form e.g.  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ,  $\text{CH}_3\text{COO}^-$ , whilst ions which interact strongly promote deformation of cells even in strong NaCl solutions e.g.  $\text{CNS}^-$ ,  $\text{CCl}_3\text{COO}^-$ ,  $\text{Cd}^{2+}$  and urea.

Studies on isolated cell envelopes have revealed that they also spontaneously breakdown upon removal of NaCl (Brown and Shorey, 1962; Larsen, 1962; Kushner et al, 1964). This disintegration is not enzymically mediated although it is pH and temperature dependant, (Brown, 1963; Kushner, 1964; Onishi and Kushner, 1966). However,  $\text{K}^+$  is almost as effective as  $\text{Na}^+$  in protecting intact cell envelopes, and  $\text{Mg}^{2+}$  is much more effective in preserving cell envelopes than whole cells.

Electron microscopy of isolated cell envelopes of H. halobium (Stoekenius and Rowen, 1966, 1967) and H. salinarium (Steensland and Larsen, 1969) has revealed a unit-membrane like structure corresponding to the cell membrane (Salton, 1967) covered on the

outer surface of the cell with protein coat appears in electron micrographs as an array of hexagonal sub-units about 130 Å in diameter (Larsen, 1967). An analysis of this layer from H. halobium has been reported by Marshall, Wicken and Brown (1969). Steensland (1967) carried out a detailed characterisation of the envelope of H. salinarium strain 1. As the  $\text{Na}^+$  content of the medium was lowered the protein coat separated from the rest of the envelope exposing the cell membrane. If  $\text{Mg}^{2+}$  concentration was also low then amino-sugars were released with the protein. Most Gram-negative bacteria contain at least some mucopolysaccharide in their envelopes (Salton, 1964) and the amino-sugars may represent part of this component. Mjelde (1968, 1969) has identified the presence of glucosamine and one other amino-sugar linked to a peptide chain. However, muramic acid, diaminopimelic acid and D-amino acids frequently present in mucopolysaccharide from both Gram positive and negative organisms have not been detected in Halobacteria species, (Brown and Shorey, 1963; Kushner et al, 1964; Steensland, 1965; Kushner and Onishi, 1968).

Since the outer protein coat of Halobacterial cell walls "dissolves" under conditions of low salinity it is possible to isolate cell membrane free from cell wall components. Comparatively pure preparations of cell membrane have been prepared from Halobacteria species (Steensland and Larsen, 1969; Stoekenius and Kunau, 1968). The procedures essentially involve exposing cell

envelopes to distilled water and isolating membrane fragments from contaminating cell envelopes and cytoplasmic components by differential centrifugation. In H. salinarium strain 1 the cell membrane is the only membrane present, but in H. halobium the situation is complicated by the presence of cell membrane, mesosomal membrane, gas vacuole membrane and so-called "purple" membrane. Thus the organisation of H. salinarium strain 1 permits the adoption of a relatively straightforward procedure for the isolation of pure cell membrane preparations.

Aspects of the biochemistry of extreme halophilism: The internal salt content of Halobacterial cells approaches the concentration of salt in the medium (Gibbons and Baxter, 1953; Christian and Ingram, 1959; Holmes, 1964). However, in addition to  $\text{Na}^+$  and  $\text{Cl}^-$ ,  $\text{K}^+$  is a dominating component of intracellular salt and its concentration approaches the saturation limit of KCl (Christian and Waltho, 1962). The concentration difference of  $\text{Na}^+$  across the cell envelope is about equal to that of  $\text{K}^+$ , but of opposite sign. Since the intracellular salt concentration is high, it is not surprising that the enzymes present are also halophilic (Baxter and Gibbons, 1954, 1956 and 1957) exhibiting optimal activity in levels of NaCl or KCl comparable to the intracellular content. Significantly many intracellular enzymes show higher activities in KCl than NaCl (Larsen, 1967) whilst certain extracellular proteases from H. salinarium have a higher

activity in NaCl than KCl, (Norberg and Hofstein, 1969). As well as stimulating halophilic enzymes high levels of salt are essential for their stability. Baxter and Gibbons (1956 and 1957) and Holmes and Halvorson (1963 and 1965b) have demonstrated that on removal of salt the majority of halophilic enzymes are denatured. In certain cases partial or complete restoration of activity can be achieved by exposing salt-free enzyme to appropriate concentrations of salt under carefully controlled conditions.

Membrane bound enzymes investigated show extremely sensitive halophilic responses e.g. NADH dehydrogenase, (Larsen, 1967), NADH oxidase (Hochstein and Dalton, 1968) and menadione reductase (Lanyi, 1969). Stevenson and Brown (1967) claim to have partially reactivated an ATPase from a salt-free state. However, membrane bound enzymes seem to be very much more sensitive to loss of NaCl than cytoplasmic enzymes and in general require higher levels of NaCl, approximately 25%, for optimum activity. This level corresponds to the response of whole cells in maintaining their rod shape and active growth.

Certain essential metabolic activities are associated with bacterial cell membranes e.g. respiratory chain (Salton 1964), ion transport (Kabach and Stadtman, 1966) and possibly protein synthesis, (Butler, Crathorn and Hunter, 1958). In the Halobacteria, Stevenson (1966) and Brown (1966) have shown salt



requirements for an active transport system and possibly oxidative phosphorylation associated with the cell membrane of H. salinarium strain 1. In connection with an investigation of protein synthesis Bayley and Kushner (1964) have demonstrated that isolated ribosomes of H. cutirubrum are halophilic, disaggregating in solutions which are less than 4M KCl and 0.1-0.4 M Mg.

Thus the Halobacterial cell membrane probably exists in a highly polar environment. This may have far-reaching effects on the interactions maintaining the integrity of the membrane. For example, polar interactions may be minimised through a shielding effect by the surrounding highly ionic medium and hydrophobic or non-polar interactions may take on a prominent role. Under these circumstances the nature of the protein-protein and protein-lipid interactions within the membrane may represent an adaptation to the halophilic mode of life, in which case one might expect to find hydrophobic interactions of greater significance than in the membranes of non-halophilic organisms.

The investigation undertaken in the work described in this thesis attempted to probe the nature of the cell membrane and selected enzymes of H. salinarium strain 1 and relate their structure to the halophilic character of the organism.

## Chapter II: The halophilic character of *H. salinarium* strains 1 and 1M

### Introduction

Professor H. Larsen has produced a colourless mutant (strain 1M) from *H. salinarium* strain 1 by prolonged U.V. irradiation. He has demonstrated that the colourless mutant, which lacks carotenoid in the cell membrane, is very much more sensitive to irradiation by high intensity Tungsten lamps. In the presence of suitable dyes cells of the mutant lyse, probably due to photochemical damage of the cell envelopes (Dundas and Larsen, 1962, 1963). Under normal light intensities the mutant is not affected.

Since carotenoid is located in the cell membrane of strain 1 (Brown, Bellingham and Stevenson, 1971) and constitutes approximately 0·1% of the cell envelope it is possible that the character of the mutant membrane is different from the wild-type. The cell membrane is the site of key metabolic processes and certain enzymes associated with the membrane of strain 1 have been shown to be markedly halophilic (see Chapter I). Thus a change in the membrane components may affect the response of the organism to variations of NaCl in the growth medium tending to a greater or lesser degree of halophilic character.

The sensitivities of the mutant and wild-type to variations of NaCl concentration were investigated by comparing their growth in culture media of different NaCl content and establishing the

levels and salt responses of selected membrane bound and cytoplasmic enzymes.

## Section I: Methods

2.1 The micro-organisms: Cultures of Halobacterium salinarium strain 1 and 1M and H. cutirubrum were gifts from Professor H. Larsen of the Technical University of Norway.

2.2 Cultivation of the organisms: H. cutirubrum and both strains of H. salinarium were kept in a lyophilised state. From time to time during the course of the project fresh vials were opened and transferred to liquid medium to ensure that the original strains were cultivated throughout the work.

Cultures for the routine growth of the organisms, investigation of enzyme levels and the preparation of cell membranes contained the following percentage (w/v) composition made up in tap-water:-

Bacteriological Peptone (Oxoid)	1%
Sodium chloride	25%
Potassium chloride	0.5%
Ammonium chloride	0.5%
Magnesium chloride	0.5%

Liquid cultures for routine growth and inocula were grown in 500 ml conical flasks containing 100 ml of medium on a Gallenkamp orbital shaker at 37°C and 200 r.p.m. In the early stages of the project cultures of 1 l. were grown in 5 l. flasks rotated at 500 r.p.m. in a thermostatted cabinet. However, due to a major

breakdown of this equipment, the later stages of work were completed using cultures grown in 2 l. conical flasks containing 400 ml of medium and agitated at 200 r.p.m. in a Gallenkamp orbital shaker at 37°C. When a 10% exponential phase inoculum was used both of these methods gave comparable cell yields of about 5 g per l wet weight after 60 hours of incubation. The presence of  $\text{NH}_4\text{Cl}$  in the growth medium (Onishi et al, 1965) and the use of a 10% inoculum (Holmes et al, 1965) reduce the duration of the lag phase.

2.3 Estimation of cell growth: Growth of the cultures was followed by measuring the turbidity of the medium using an E.E.L. photometer with a 608 filter.

2.4 Determinations of salt-free dry weight: Aliquots of washed cells in preweighed silica crucibles were heated to constant weight in an oven at 95°C. This residue was then heated in a furnace at 800°C for 8 hours to remove all organic matter. The final salt residue was weighed to obtain the salt-free dry weight.

2.5 Protein determination: Protein was estimated by the Folin - Ciocalteu method (Lowry, Rosebrough, Farr and Randall, 1951) using bovine serum albumin as a standard. Whole cells and cell envelopes were solubilised in 0.2% sodium deoxycholate.

2.6 Estimation of enzyme levels:

Harvesting of cultures: Cells were separated from the culture medium towards the end of exponential growth by centrifuging at

5000 x g for 10 minutes in a 6 x 250 ml rotor of an M.S.E. Mistral 6L centrifuge previously cooled to 0°C. The pellet of cells was washed by careful resuspension using a large bore pipette into a Tris-salts buffer containing 0.1M Tris HCl pH 8.0, 25% NaCl, 0.5% MgCl<sub>2</sub> and recentrifuged.

Disruption of cells:

(a) Braun glass bead homogeniser: A pellet of washed cells from 1½ l of culture was resuspended in about 15 ml of cold Tris-salts buffer. Approximately 25-30 g of ballotini (0.17 - 0.18 mm) previously washed with concentrated HCl and distilled water were then added. The homogeniser was precooled using liquid CO<sub>2</sub> and a run of 1 minute at full speed was sufficient to break the cells. The resulting suspension was diluted 1:1 with cold Tris-salts and spun at 5000 x g. for 10 minutes at 0°C to remove unbroken cells and ballotini. The supernatant contained cell fragments and intracellular contents.

(b) Sonication: 5 ml samples of washed cells containing about 0.5 g wet weight of cells suspended in cold Tris-salts buffer were sonicated for 4 x 15 seconds using an M.S.E. sonicator with a chilled probe. Unbroken cells were removed by centrifuging at 5000 x g for 10 minutes at 0°C.

(c) Teflon homogeniser: About 1 g wet weight of cells in 10 ml of cold Tris-salts was homogenised by 4-5 passes with a

Potter - Elvehjem homogeniser. The whole operation was carried out at 4°C in a cold room. Unbroken cells were removed by centrifuging at 5000 x g for 10 minutes at 0°C.

Centrifugation of cell fragments: Suspensions of the cell fragments obtained by the methods described above were centrifuged at 27,000 x g for 1 hour at 0°C using an M.S.E. high speed centrifuge with a 6 x 100 ml rotor. The pellet of cell envelopes obtained was resuspended and washed twice with cold Tris-salts buffer. The supernatant from the first centrifugation was spun at 150,000 x g for 2 hours at 0°C using a Beckman Model L-2 ultracentrifuge to remove any small fragments of cell envelope or membrane.

Assay of enzymic activity:

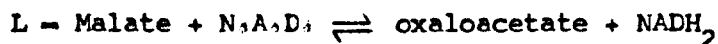
Adenosine triphosphatase: The ATPase activity of preparations was measured by determining the amount of inorganic phosphate released from ATP. For each assay two tubes were prepared containing the following reagents:-

0.1M ATP	0.1 ml
0.1M Tris. HCl pH 8.0	4M NaCl 1.15 ml
0.8M MgCl <sub>2</sub>	0.25 ml
Enzyme preparation	0.5 ml

The reaction tube was incubated at 37°C for 1 hour and then

stopped by the addition of 2.0 ml of cold 10% trichloroacetic acid (T.C.A.). The control tube was treated with T.C.A. prior to the addition of enzyme. Both tubes were kept in ice for 30 minutes and then precipitated protein was removed using a bench centrifuge. A 1.0 ml aliquot of each supernatant was then assayed for inorganic phosphate by the method of Fiske and Subba Row (1925).

Malate dehydrogenase (MDH); Malate dehydrogenase catalyses the following reaction:



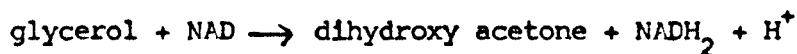
Activity was assayed by following the conversion of oxaloacetate to malate via the concomitant utilisation of  $NADH_2$ . The cuvette contained the following reagents equilibrated at 30°C:-

0.07M KCN	0.05 ml
0.07M KF	0.05 ml
0.1M iminazole pH 7.75. 4M KCl	2.2 - 2.6 ml
0.03M oxaloacetic acid	0.35 ml
1 mM $NADH_2$	0.35 ml

The reaction was started by the addition of up to 0.5 ml of enzyme and the rate of change of absorbance at 340 nm was measured using a Gilford 2000 spectrophotometer.

Glycerol dehydrogenase (GDH): The enzyme catalyses the reaction:-





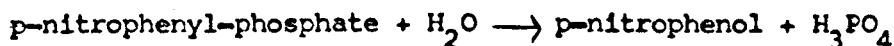
The activity of preparations were measured by following the conversion of NAD to  $\text{NADH}_2$ .

The reaction cuvette contained the following reactants equilibrated at  $30^\circ\text{C}$ :-

1.9 - 1.95 ml KCl	4.0 <u>M</u>
0.05 ml KCN	0.07 <u>M</u>
0.05 ml KF	0.07 <u>M</u>
1.0ml NAD	1 <u>mM</u> in 0.01 <u>M</u> iminazole
	pH 7.75
0.35 ml glycerol	1 <u>M</u>

The reaction was started by the addition of 0.1 - 0.5 ml of enzyme and the rate of increase in absorbance at 340 nm measured.

Phosphatases: Both acid and alkaline phosphatase activity may be estimated colourimetrically by measuring the rate of hydrolysis of p-nitrophenyl-phosphate:-



(colourless in acid and  
alkali)

(colourless in acid and yellow  
in alkali)

(1) Alkaline phosphatase: The cuvette contained the following reactants equilibrated at  $30^\circ\text{C}$ :

2.4 - 2.8 ml. 0.1M Glycine pH 10.0, 4M KCl

0.1 - 0.5 ml. Enzyme preparation

Total volume 2.9 ml

This mixture was used to zero the instrument and the reaction was started by the addition of 0.1 ml p-nitrophenyl phosphate (4 mg/ml). The rate of increase of absorbancy at 410 nm was measured using a Gilford spectrophotometer.

(ii) Acid phosphatase: For each assay 3 tubes were set up as follows:-

Reagent	Reaction tube (ml)	Reagent blank (ml)	Protein blank (ml)
0.05M sodium citrate pH 5.2 + 4M NaCl	0.9	0.9	-
p-nitrophenyl phosphate (10 mg/ml)	0.1	0.1	-
Enzyme preparation	0.5	-	0.5
Water	-	0.5	-
Each tube was incubated at 37°C and after 30 mins stopped by the addition of 0.1M NaOH	5.0	5.0	6.0

The absorbance at 410 nm of the Reaction tube and the Protein blank were measured against the Reagent blank using a Zeiss PMQII spectrophotometer. The acid phosphatase activity was then determined as micromoles p-nitrophenol released by reference to a standard curve.

## Section II: Results

### 2.7 Effect of salt concentration on the growth in liquid culture of *H. salinarium* strains 1 and 1M

The cultures were grown in 500 ml flasks fitted with side-arms and containing 100 ml of medium. Two types of media were tested, one contained the normal proportion of constituents, as described in paragraph 2.2, whilst in the other 25% NaCl and 0.5%  $\text{MgCl}_2$  were replaced by 20% NaCl and 2%  $\text{MgCl}_2$ . The level of  $\text{Mg}^{2+}$  in the low salt medium was increased because  $\text{Mg}^{2+}$  has been shown to maintain the shape of halobacterial cells as NaCl concentration is reduced (see Chapter I).

The cultures were started with 1% inocula of exponential phase cultures and incubated at 37°C on a Gallenkamp shaker. 1% inocula were used so that any differences in the lag phases could be detected. Periodically the turbidities of the cultures were measured using an E.E.L. colorimeter.

The growth curves obtained are illustrated in Figures 2.1a and 2.1b. In addition Figure 2.2 shows a growth curve recorded by measuring the salt-free dry weights of 5 ml aliquots taken during the growth cycle of cultures of strain 1 and 1M in 25% NaCl and 0.5%  $\text{MgCl}_2$  media started with 10% inocula.

Figure 2.1

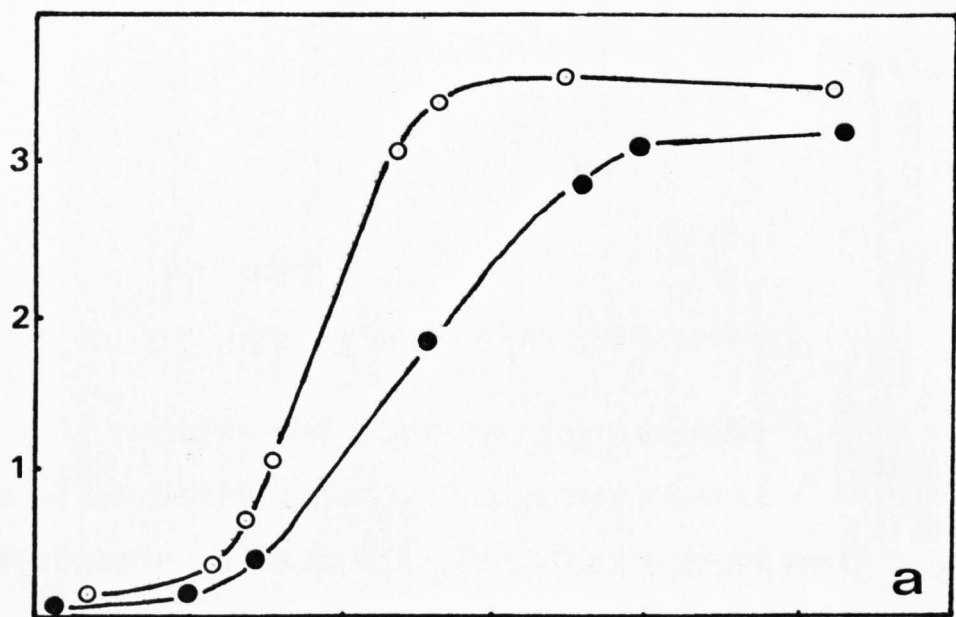
Growth characteristics of H. salinarium strains 1 and 1M

Cultures were started with 1% inocula and incubated at 37°C in Gallenkamp orbital shakers. Turbidities of cultures were measured periodically with an E.E.L. colourimeter (608 filter).

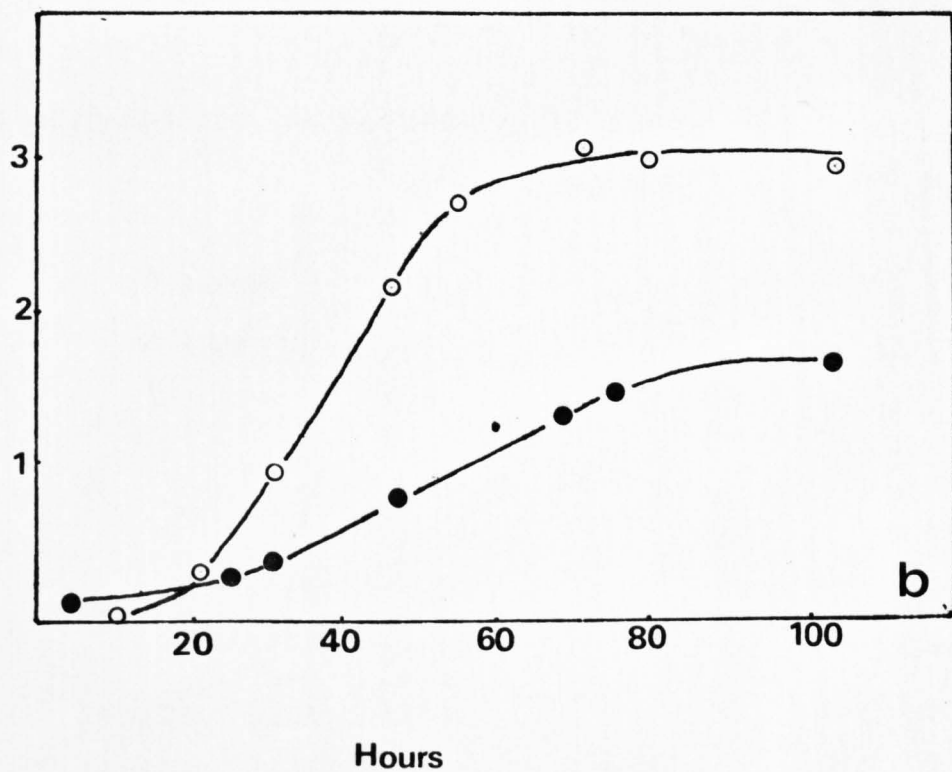
- (a) Medium as in paragraph 2.2 i.e. containing 25% NaCl and 0.5%  $MgCl_2$ .
- (b) Medium as in paragraph 2.2. but containing 20% NaCl and 2%  $MgCl_2$ .

○ strain 1

● strain 1M



Turbidity



Hours

Figure 2.2

Growth characteristics of *H. salinarium* strains 1 and 1M

Cultures were started with 10% inocula and incubated at 37°C in Gallenkamp orbital shakers. Growth media were as described in paragraph 2.2 containing 25% NaCl and 0.5% MgCl<sub>2</sub>. Growth was determined by periodically measuring salt-free dry weights of cell mass (g/l of culture).

○ strain 1

● strain 1M

Salt-free dry wt. of cell mass, g per l of culture

2

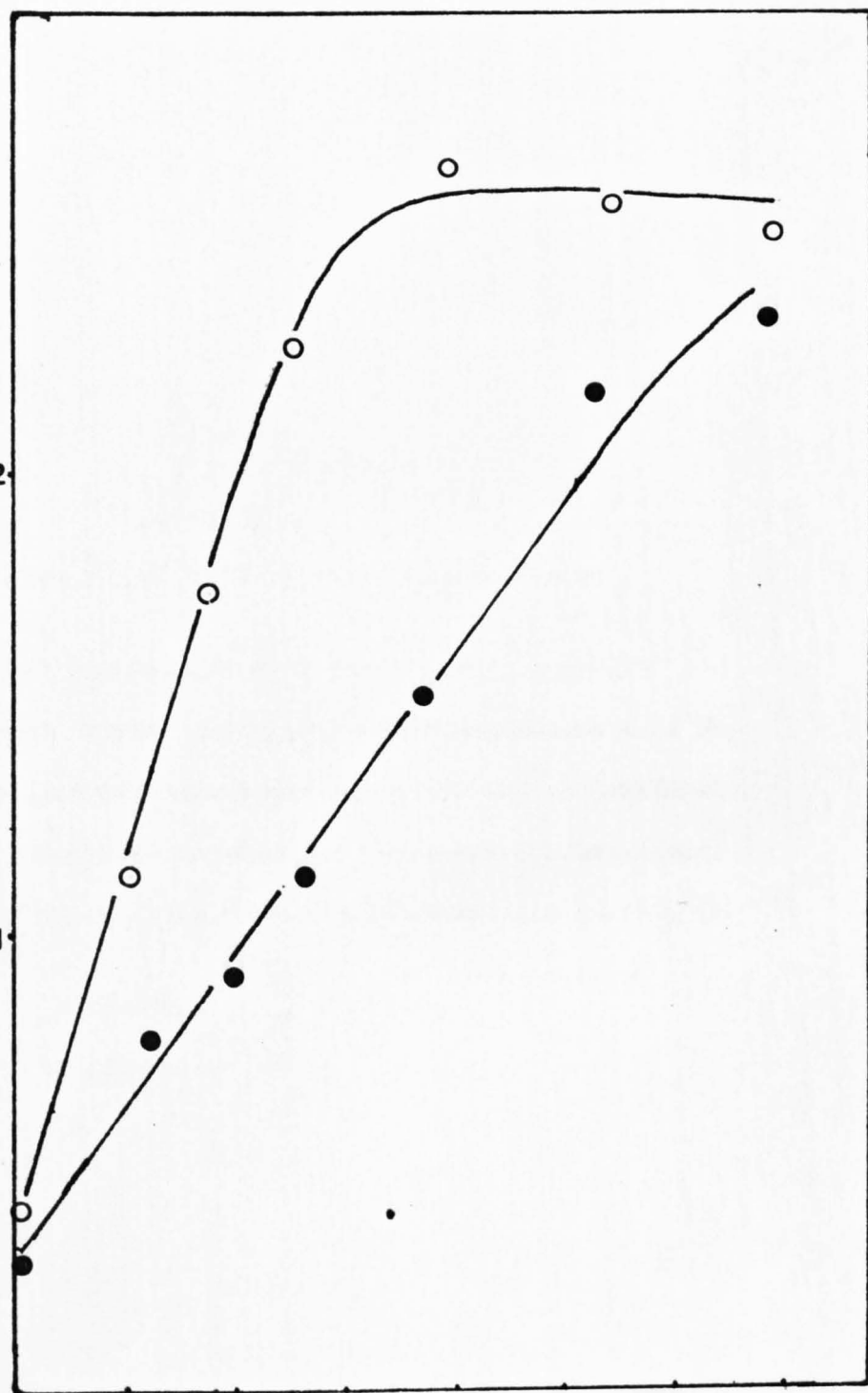
1

32

64

96

Age of culture, hours





## 2.8 The estimation of enzyme levels in strain 1 and 1M

Mid-logarithmic phase cells of strains 1 and 1M H.salinarium were disrupted by glass bead homogeniser, sonication and teflon homogeniser. The cell homogenate from each of these methods was then separated into a cell-envelope and supernatant fraction as described in Section I of this chapter. Total activity was estimated from the cell homogenates diluted with cold Tris-salts buffer as necessary.

Table 2.1 summarises the percentage distributions of activities obtained using the different methods of disruption. Table 2.2 shows the specific activities obtained in the most active preparation of each enzyme for both strains.

Enzyme		Percentage Distribution			
		Cell Envelope		Supernatant	
		Strain 1	1M	1	1M
ATPase	a)	100	100	0	0
	b)	100	100	0	0
	c)	100	100	0	0
Acid phosphatase	a)	76	70	24	30
	b)	73	70	30	26
	c)	80	71	16	16
Alkaline phosphatase	a)	0	<5	98	90
	b)	0	0	100	100
	c)	0	0	98	95
MDH	a)	<1	<10	95	90
	b)	0	0	100	100
	c)	0	0	100	98
GDH	a)	0	0	100	100
	b)	0	0	100	100
	c)	0	0	100	100

Table 2.1 Distribution of enzymic activities in cell envelope and solubilised fractions obtained from disrupted cells of H. salinarium strains 1 and 1M. Details of preparation and assay methods are given in the text of paragraph 2.6. Total activities were determined on unfractionated broken cell homogenates. Methods of cell disruption

- a) glass bead homogeniser
- b) sonication
- c) teflon homogeniser

Enzyme	Fraction	Specific activity		Method of Cell Disruption
		Strain 1	Strain 1M	
ATPase	cell envelope	0.46	0.44	glass beads
Acid phosphatase	cell envelope	0.2	0.1	glass beads
Alkaline phosphatase	supernatant	0.5	0.05	sonication
MDH	supernatant	0.4	0.1	sonication
GDH	supernatant	0.2	0.2	sonication

Table 2.2 Levels of selected enzymes in the most active fractions from disrupted cells of H. salinarium strains 1 and 1M. Units of specific activity are as follows:

ATPase:  $\mu$  moles Pi released/hour/mg protein

Acid phosphatase:  $\mu$  moles p-nitrophenol released/min/mg protein

Alkaline phosphatase:  $\mu$  moles p-nitrophenol/min/mg protein

Malate dehydrogenase (MDH):  $\mu$  moles NAD/min/mg protein

Glycerol dehydrogenase (GDH):  $\mu$  moles NADH/min/mg protein

2.9 The effect of NaCl and KCl on the activities of enzymes from strains 1 and 1M

The enzymes investigated were ATPase and acid phosphatase, both associated with the cell envelope fractions in strain 1 and 1M, and alkaline phosphatase and glycerol dehydrogenase which were present in the supernatant fractions (see results in Table 2.1).

10 ml aliquots of the cell envelope and supernatant fractions were dialysed for 48 hours at 4°C against several changes of 1 l volumes of 0.1M Tris. HCl pH 8.0 containing NaCl or KCl in the range 1.0M - 4.0M. The dialysates were assayed for each enzyme as described in Section I, but in the presence of concentrations of NaCl or KCl corresponding to the levels of these salts in the dialysis buffers. Figures 2.3 (a), (b), (c) and (d) illustrate the results of the assays.

Figure 2.3

Salt responses of selected enzymes associated with the cell envelopes and cytoplasmic contents of H. salinarium strains 1 and 1M.

Aliquots of the enzyme preparations were dialysed against 0.1M Tris. HCl pH 8.0 containing NaCl or KCl in the range 1.0 - 4.0M. Assays were carried out in the same buffer containing corresponding levels of salt. Results were expressed as a percentage of the maximum activity obtained.

Enzyme assays:-

Cell envelope:

- (a) ATPase
- (b) Acid phosphatase

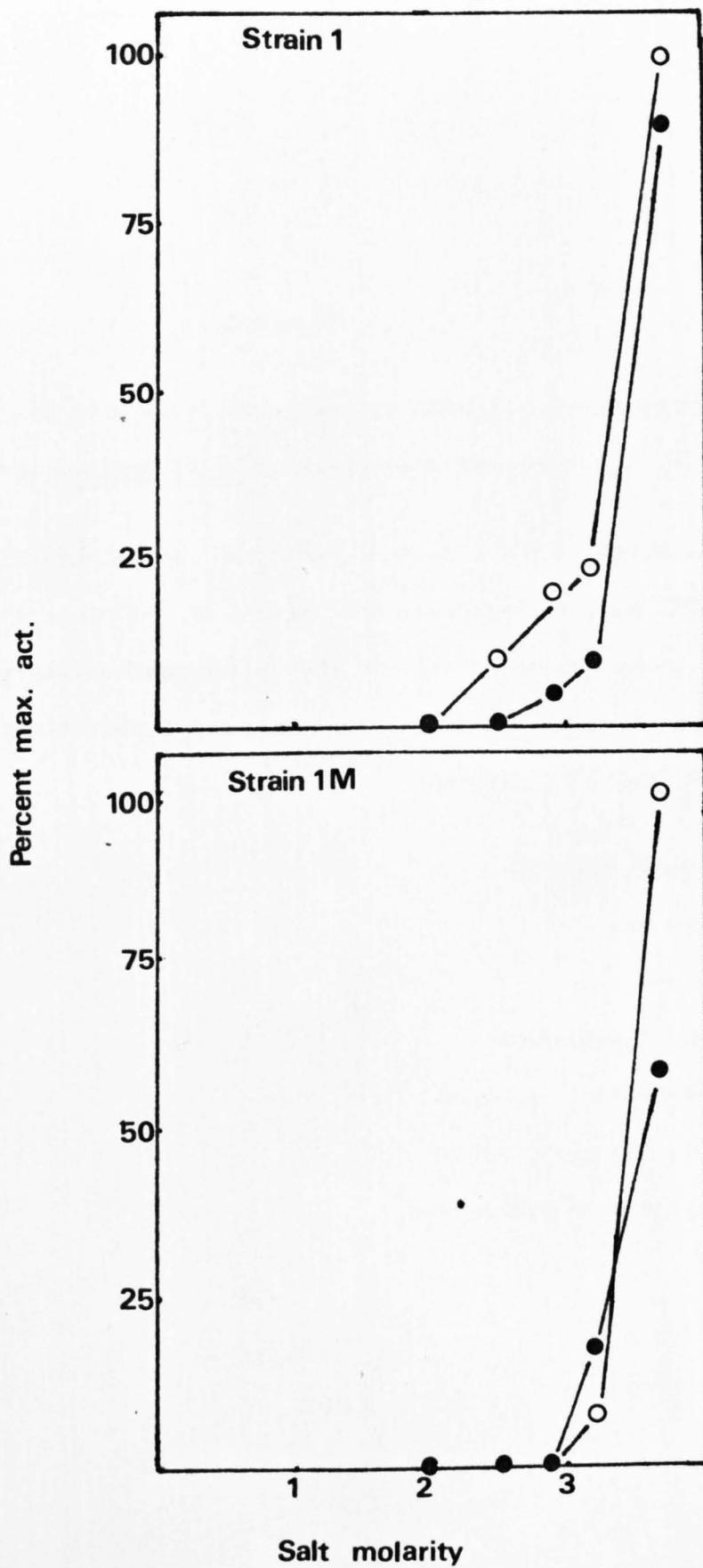
Cell supernatant fraction:

- (c) Alkaline phosphatase
- (d) Glycerol dehydrogenase

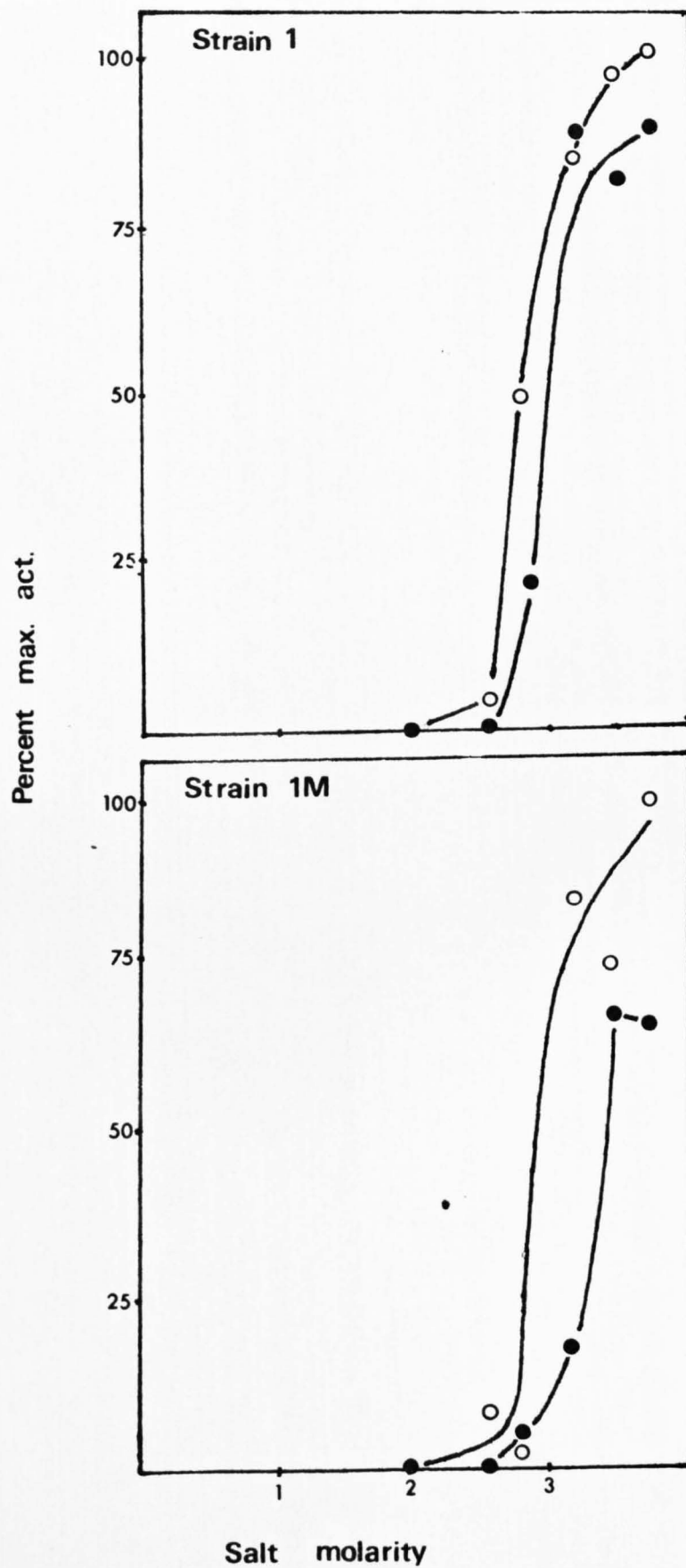
○ NaCl

● KCl

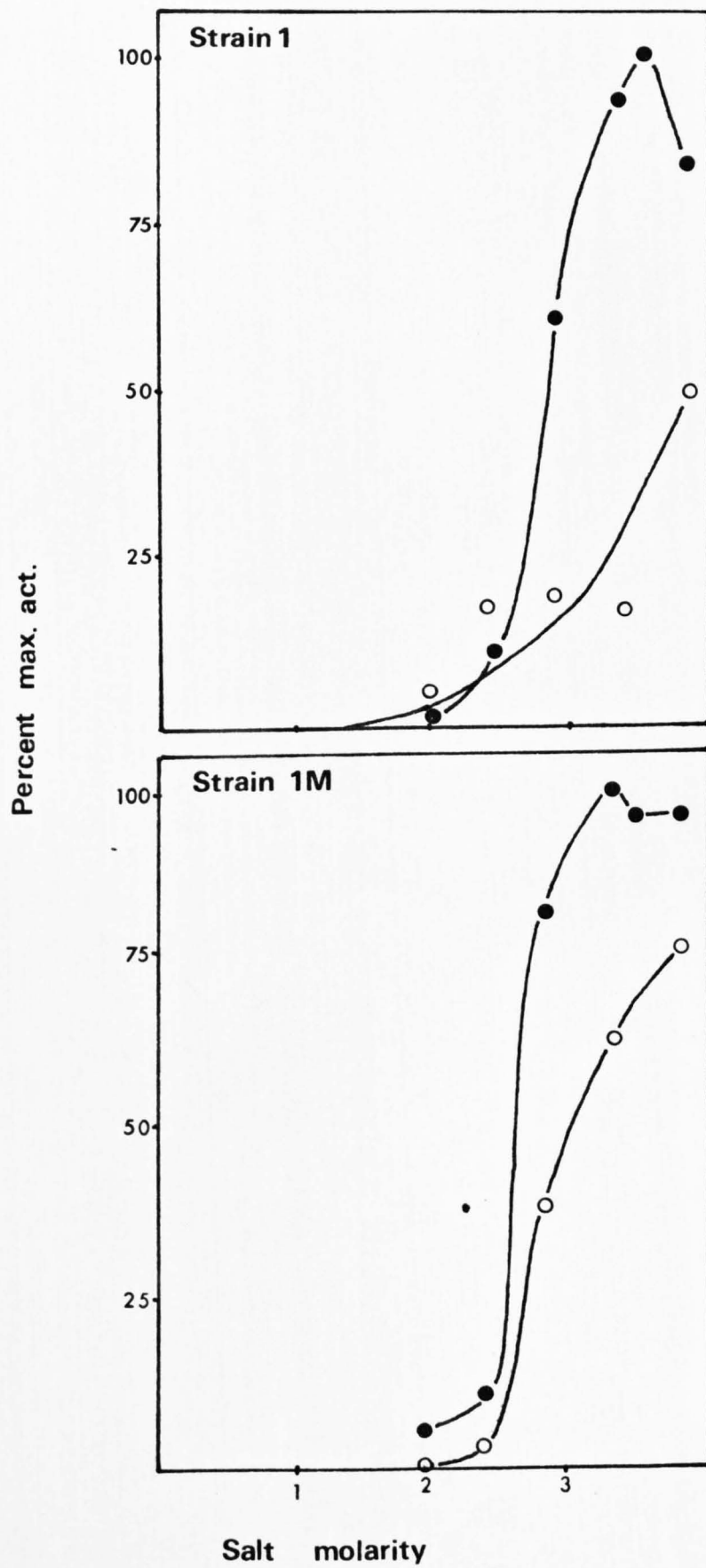
a



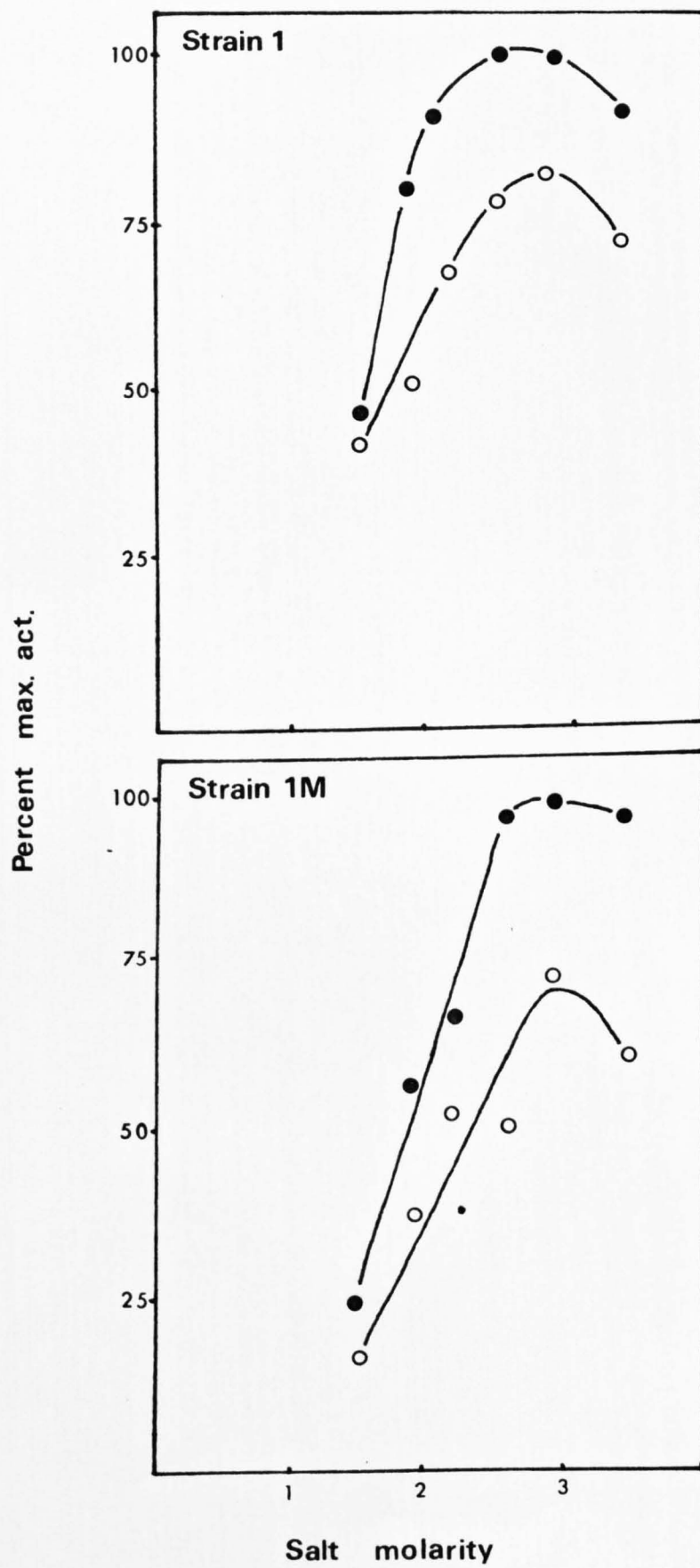
b



C







d

### Section III    Discussion

The growth of both wild-type (strain 1) and mutant (strain 1M) was affected by a reduction in the level of NaCl in the culture medium. (see Figure 2.1). The rate of growth in the medium containing less salt tended to be slower, in both cases taking about 9% longer to reach the end of log. phase, and the final yields of cells as estimated by turbidometry were lower. However, in strain 1 growth was reduced to a lesser extent than in the case of the mutant. In 20% NaCl medium the mutant suffered a 48% reduction of maximum growth attained, whilst strain 1 was reduced by only 17%. These results indicate that under the growth conditions employed the mutant strain was more halophilic than the wild-type.

Disruption of the cells by glass-bead homogeniser gave more active preparations of membrane-bound enzymes, whilst sonication gave better yields of cytoplasmic enzymes. Sonication may well have significantly altered a proportion of the cell envelope structure and thereby inactivated membrane bound enzymes. In general the levels of the enzymes tested were slightly lower in the mutant preparations than in strain 1 (see Table 2.2). Certain enzymes were significantly less active, i.e. envelope bound acid phosphatase showed a 50% reduction in activity in mutant preparations and MDH activity in the solubilised fraction was 25% lower than in strain 1. The greatest difference was in alkaline phosphatase which

was 90% less active in the mutant. However, respiratory chain activities in envelope preparations of either strain were found to be comparable (W.E. Lancashire - private communication).

The envelope bound enzymes investigated were very sensitive to changes of salt concentration (see Figure 2.3). Mutant ATPase activity was marginally more sensitive than strain 1. The cytoplasmic enzymes from both strains showed much greater tolerance of lower salt concentrations, than membrane-bound enzymes, especially in the presence of KCl. Unlike the bound enzymes, cytoplasmic enzymes gave higher activity in the presence of KCl than NaCl. These results are in general in agreement with the findings reviewed by Larsen (1967) for H. salinarium and other Halobacteria.

The overall picture emerging is that the enzymes of both strains 1 and 1M are of comparable halophilic character showing optimal activity in levels of salt corresponding to those of the growth medium or the intracellular content. However, the membrane-bound enzymes are more halophilic than the cytoplasmic enzymes. Possibly the removal of NaCl may affect the membrane, the bound enzyme, or both, leading to conformational changes which inactivate the enzyme.

Chapter III     The purification of malate dehydrogenase and  
investigation of structural changes due to variations  
of salt concentration

Introduction

Baxter (1959) and Holmes and Halvorson (1965a) investigated the reversible inactivation undergone by halophilic enzymes on removal of salt. Under carefully controlled conditions of dialysis salt was removed and then returned to enzyme extracts. The reactivation process was slow, taking considerably longer than the time required for the salt to establish equilibrium between the inside and outside of the dialysis bag. They suggested that this observation implied a slow conformational rearrangement of the salt-free enzyme induced by the presence of salt.

It is generally accepted that most proteins in their native state exist as rather tight compact structures folded to give highly specific conformations. (Schachman, 1963a). This is particularly relevant to enzymes where slight alterations of tertiary structure can lead to total loss of biological activity. Measurements of viscosity, frictional coefficients and light scattering characteristics can provide information on the general shape of macro-molecules in solution (Schachman, 1963b; Tanford, 1961; Djerassi, 1960). However, X-ray diffraction studies are required to obtain a detailed determination of molecular structure.

This technique used in conjunction with amino acid sequence analysis has been applied successfully to several crystalline proteins, e.g. myoglobin (Kendrew et al., 1960, 1961) and lysozyme (Blake et al., 1965). However, it is not always practical to study protein structure by crystallographic methods. Optical techniques such as absorption spectroscopy, fluorescence spectroscopy, optical rotatory dispersion and nuclear magnetic resonance spectroscopy are useful in detecting the subtle changes of conformation which proteins may undergo in solution, (Mahler and Cordes, 1966).

Fluorescence spectroscopy is one of the most sensitive and versatile optical techniques for studying protein structure. Most proteins contain tryptophan and tyrosine residues which will fluoresce when excited by ultra-violet light. The emission may change in intensity, polarisation or wavelength under the influence of small changes of protein structure which may result from interactions with other molecules. Following the original work of Weber (1960) and Teale (1960) several workers have exploited the characteristics of intrinsic fluorescence to study protein structure (Yamari and Bovey, 1960; Wetlaufer, 1962). However, the residues of a given protein may not give a strong enough response. Moreover, the parameters within the protein molecule influencing intrinsic fluorescence are difficult to analyse and consequently it may be necessary to add environmentally sensitive fluorescent molecules of known properties as probes of protein structure. (Horton and

Koshland, 1967; Edelman and McClure, 1968).

Of the compounds used as fluorescent probes three have been particularly well investigated:- 2,6-TNS (2-p-toluidinylnaphthalene-6-sulphonate) and two isomers of ANS (1-anilinonaphthalene-4-sulphonate and 1-anilinonaphthalene-8-sulphonate). These compounds fluoresce more strongly in nonpolar than polar solvents. They are virtually non-fluorescent in water but their intensity of fluorescence increases markedly when dissolved in organic solvents or when bound to proteins (Weber and Laurence, 1954; Stryer, 1965; McClure and Edelman, 1966, 1967a and 1968). Thus in aqueous solutions of proteins fluorescence due to unbound probe molecules is minimal.

ANS has been used for structural studies on several proteins, (Stryer, 1965; Weber and Young, 1964; Anderson and Weber, 1966), for detection of conformational changes in proteins, (Dodd and Radda, 1967; McClure and Edelman, 1967a) and for following the rate of protein conformational changes, (Dodd and Radda, 1969). McClure and Edelman (1967b) have used the probe TNS to follow the conformational change which occurs on the formation of chymotrypsin from its inactive precursor chymotrypsinogen (Neurath et al., 1956). The probe responded to the subtle structural changes of the enzyme proteins caused by the refolding of a polypeptide chain which accompanied the formation of the active site.

Small structural changes of this nature may be involved in the reactivation of salt-free halophilic enzymes. The experiments described in this chapter were carried out to investigate the possibility of using 1,8-ANS as a fluorescent probe of halophilic proteins.

Malate dehydrogenase, was studied because a partial purification of the enzyme had previously been achieved by Holmes and Halvorson (1965b). A pure protein is essential for fluorescent labelling experiments and the purification procedure was improved by the use of isoelectric focusing, a technique which has recently found wide application for enzyme purification (Haglund, 1970).

## Section I : Methods

3.1 Cultivation of the organism: H. salinarium strain 1 was grown in 15 litres of peptone-salts medium (see paragraph 2.2) in a 20 litre Quickfit fermentation vessel stirred rapidly with a magnetic stirrer and heated at 37°C with a Churchill water circulator connected to a glass coil which was immersed in the culture medium. Air was supplied through a glass sinter at a rate of 0.6 cu. ft./minute and excessive foaming was eliminated by the addition of 4.0 ml of Silicone MS Antifoam A (Hopkins and Williams).

The culture was started with a 1% log-phase inoculum and harvested after 66 hours growth using a refrigerated Sorval RC2-B centrifuge fitted with a continuous flow head. A wet cell yield of approximately 8 g/l was obtained.

### 3.2 Preparation of crude extract of malate dehydrogenase (MDH):

The harvested cells were washed with Tris-salts buffer and divided into aliquots of 15 ml containing about 10 g wet weight of cells. Each cell suspension was then disrupted using a Braun glass bead homogeniser as described in paragraph 2.6, and the supernatant was freed from glass beads and cell envelopes by centrifuging at 5000 x g for 10 minutes and 27,000 x g for 45 minutes in a Sorval centrifuge at -10°C using a GSA rotor.

### 3.3 Purification of MDH crude extract: The method of purification



was based upon that described by Holmes and Halvorson (1965b).

Stage I: Acetone precipitation: Crude extract containing 15-20 mg protein per ml was chilled to  $-20^{\circ}\text{C}$  in a deep freeze and treated with 0.5 volumes of acetone, also at  $-20^{\circ}\text{C}$ . The acetone was added at about 10 ml per minute with continual magnetic stirring. The mixture was left at  $-20^{\circ}\text{C}$  for about 30 minutes before centrifuging at  $15,000 \times g$  for 10 minutes in a Sorval centrifuge at  $-15^{\circ}\text{C}$ . The resulting supernatant was treated with a further 0.5 volumes of chilled acetone and centrifuged as before. Both pellets were then dissolved in 0.01M Tris.HCl pH 8.0 and dialysed for 24 hours against two changes of 8 l of Tris.HCl to remove acetone. An aliquot from each sample was reactivated for the assay of MDH by dialysing overnight against 0.01M Tris. HCl pH 8.0 containing 25% NaCl.

Stage 2: Ammonium sulphate precipitation: Holmes and Halvorson found that the most effective way of carrying out the precipitation was to add solid ammonium sulphate buffer surrounding a dialysis bag containing the acetone fraction. This method is somewhat slow but is effective. The ammonium sulphate concentration at which the MDH was precipitated was rather variable. In order to minimise the loss of MDH activity ammonium sulphate was added in portions of 5 g per 100 ml of buffer up to a total of 20 g per 100 ml buffer and each precipitate obtained was assayed for MDH and protein. The most active were combined and retained. Prior to

reactivation and stage 3 it was essential to remove ammonium sulphate by exhaustive dialysis against 0.01M Tris. HCl pH 8.0 at 4°C. The resulting solution was concentrated using an Amicon Diaflo ultrafilter with a UM-2 membrane.

Stage 3: Diethylaminoethyl (D.E.A.E.) cellulose chromatography:

Long and short fibres were removed from an aqueous suspension of DE22 (Whatman Chromedia D.E.A.E.). The exchanger was then precycled in 0.5N HCl and 0.5N NaOH and finally washed and equilibrated with water and 0.01M Tris. HCl pH 8.0. A 25 x 1.5 cm column was prepared and equilibrated overnight at 4°C with Tris. HCl buffer at a flow rate of 20 ml per hour. A sample of the ammonium sulphate fraction containing about 10 mg of protein was applied to the column in Tris. HCl buffer and elution was carried out with stepwise increases of NaCl concentration. The column effluent was monitored at 254 nm with an LKB Uvicord and recorder and 5 ml fractions were collected with an Ultrorac fraction collector. Fractions containing individual protein peaks were combined and an aliquot from each reactivated by dialysis against 0.01M Tris. HCl pH 8.0, 25% NaCl and assayed for MDH activity.

Stage 4: Isoelectric focusing: Isoelectric focusing has been used widely as a technique for purifying proteins (Vesterberg, 1970; Haglund, 1970). The method requires the presence of special buffer compounds, carrier ampholytes, which upon electrolysis give a pH

gradient in which proteins can focus at their isoelectric points.

The technique was carried out using an LKB isoelectric focusing column of a capacity of 110 ml. The apparatus was set up as described in the LKB operating manual in a cold room at 4°C. The lower electrode was made the anode using 1% sulphuric acid as the electrode buffer. A linear sucrose gradient was layered on top of the electrode solution using an LKB gradient mixer. The gradient contained 1% Ampholine (LKB) pH range 3-10 and 5 mg of protein. The concentration of salts in the MDH fraction from the DEAE was reduced by dialysis against 5mM Tris. HCl pH 8.0 before mixing in the gradient. The upper electrode was made the cathode and surrounded by 2% ethanolamine. Power was supplied at 300 volts and 0.3 mA for 60 hours. At the end of the run the column was emptied using a peristaltic pump at a flow rate of 40 ml per hour. The effluent was collected in 3 ml fractions and the absorbancy at 280 nm was measured using a Unicam SP500 spectrophotometer. The effluent was divided into four fractions. These fractions, containing each protein peak, were combined and dialysed overnight against two changes of 0.01M Tris. HCl pH 8.0 to remove sucrose and Ampholine and then against 0.01M Tris. HCl pH 8.0 containing 25% NaCl to reactivate MDH activity.

### 3.4 Characterisation of purified MDH

Gel filtration on Sephadex G100: A quantity of Sephadex G100

(Pharmacia) was suspended in 0.01M Tris. HCl pH 8.0 containing 25% NaCl and allowed to swell for 3 days at 4°C. A 50 x 1.5 cm column was then set up and equilibrated with 0.01M Tris. HCl, 25% NaCl buffer at a flow rate of 10 ml per hour. A sample of purified MDH containing about 1 mg of protein was applied to the column and 3 ml fractions were collected using a fraction collector. The absorbancy of the fractions at 280 nm was measured with an SP500 spectrophotometer and protein containing fractions were assayed for MDH.

Polyacrylamide disc electrophoresis: The principles of the method used were described by Davis (1964). The gels consisted of a 3.5% acrylamide stacking gel prepared with Tris. HCl pH 6.7 and a running gel pH 9.5. The electrode reservoirs were filled with Tris. glycine buffer pH 8.3. Samples of MDH containing about 200 µg of protein were applied to the top of the gels and the current was adjusted to 3 mA per tube for 10 minutes, and 5 mA per tube for 90 minutes. At the end of the run the gels were fixed for 30 minutes in 20% T.C.A. and stained for protein with 0.025% Coomassie Blue as described by Chrambach et al (1967).

3.5 Fluorescence spectrophotometry: Fluorescence spectroscopy was carried out using a Farrand Mark I Spectrofluorimeter. All fluorescence intensity (If) measurements were made at room temperature with slit-widths of 20, 20, 5 and 5mm. The instrument

was set up using a perspex standard excited at 360 nm with emission analysed at 420 nm. Test solutions containing 1-anilino-napthalene-8-sulphonate (ANS) were excited at 365 nm.

## Section II : Results

3.6 Purification of MDH crude extract: The pellet obtained by the first addition of acetone contained about 10% of the original activity, large amounts of nucleic acid, and was coloured red by carotenoid which was present in the crude extract. The second pellet was pale yellow when dissolved in Tris. HCl and contained about 20% of the MDH activity and only about 5% of the original protein. The remaining supernatant contained less than 10% of the original activity. This second pellet dissolved in Tris. HCl was used for the ammonium sulphate precipitation. MDH activity was usually associated with the protein precipitated after the addition of 10-15 g ammonium sulphate per 100 ml buffer.

When this fraction was applied to the DEAE column an elution profile was obtained as illustrated in Figure 3.1. All of the MDH activity was found in the protein eluted with buffer containing 2% NaCl. The specific activity of this fraction could be further increased by isoelectric focusing. Figure 3.2 shows the result of an isoelectric focusing run. The MDH activity was associated with protein concentrating in the column at pH 5.0-6.0.

Table 3.1 summarises the results of the complete purification procedure.

Stage	Volume ml	MDH units/ml	Protein mg/ml	Specific activity	Yield %
Crude extract	800	1.3	8.7	0.15	100
Acetone ppt <sup>n</sup>	350	0.65	0.86	0.75	22
Ammonium sulphate ppt <sup>n</sup>	200	0.63	0.06	10.4	12
DEAE cellulose	20	2.5	0.17	14.7	5.0
Isoelectric focusing	5	5.9	0.12	49.2	2.8

Table 3.1 The recovery and specific activities of MDH fractions obtained at the various stages of purification.

Figure 3.1

Elution profile of a DEAE cellulose column  
used in the chromatography of crude MDH

Approximately 10 mg of protein was applied to the top of a  
25 x 1.5 cm column of DEAE cellulose equilibrated with 0.01M  
Tris. HCl pH 8.0 buffer at a flow rate of 20 ml per hour.  
Elution was carried out by stepwise increases of NaCl concentration.  
Shading shows the distribution of MDH activity.



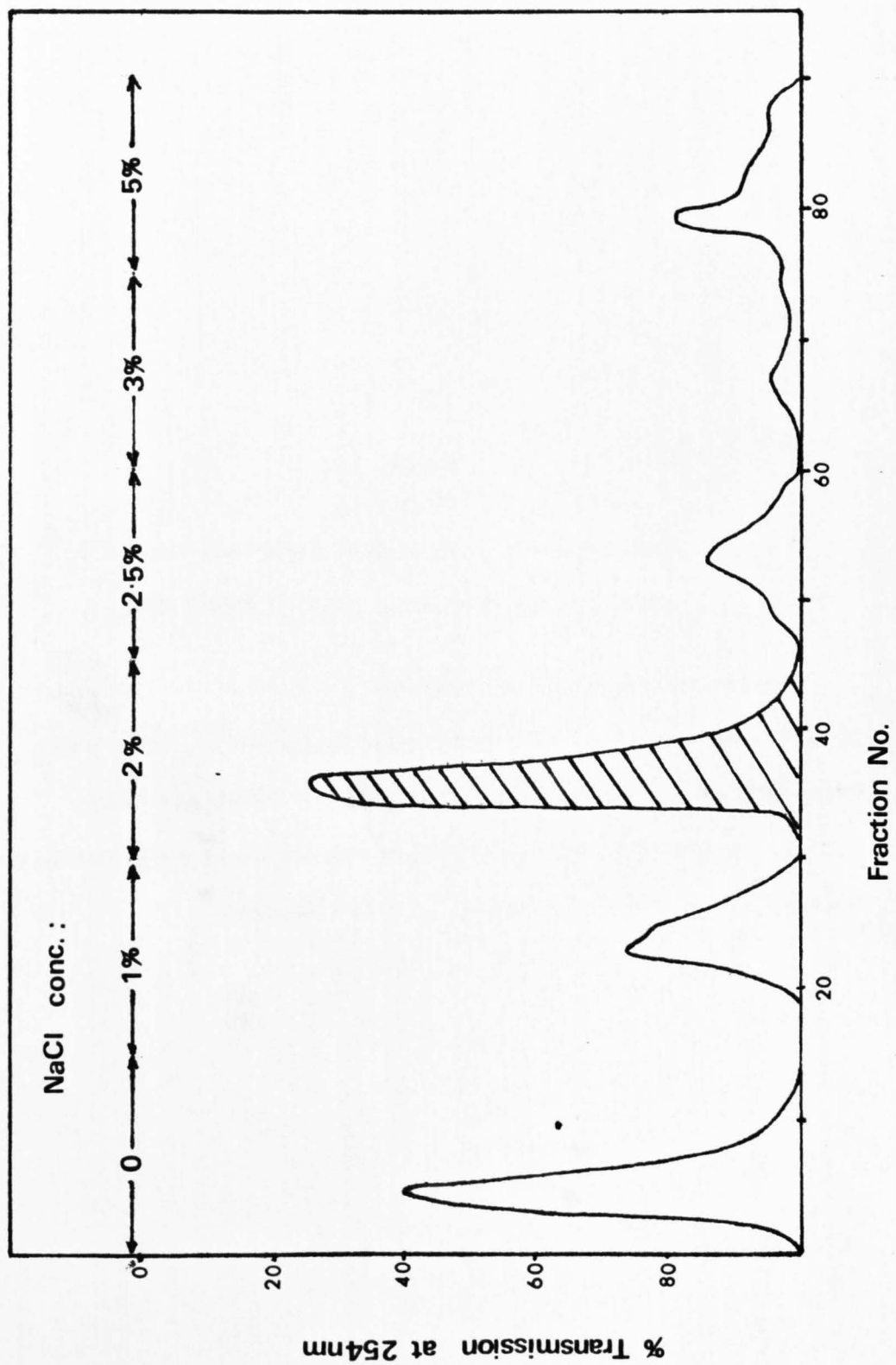


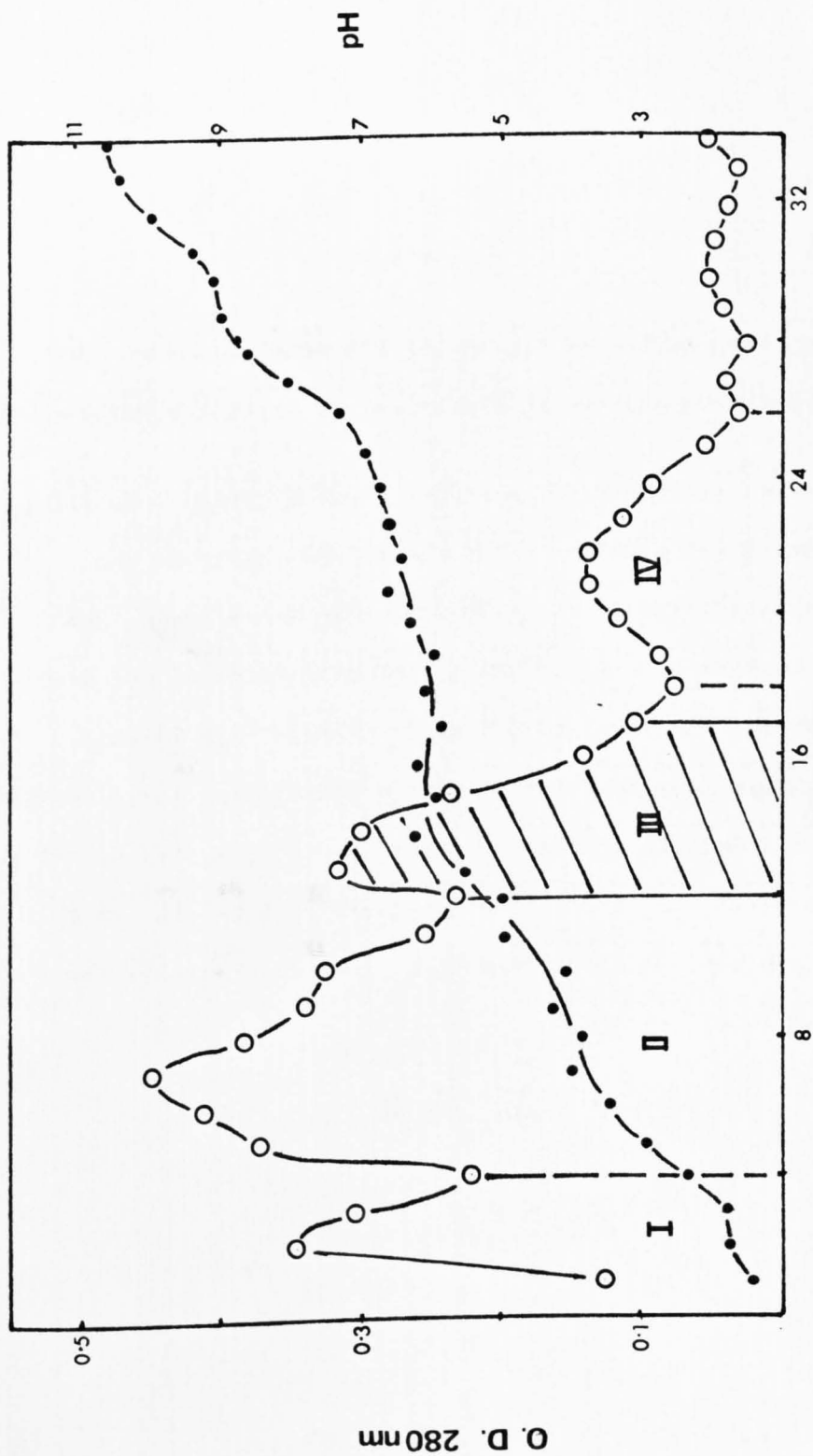
Figure 3.2

The distribution of protein in the column effluent from an isoelectric focusing separation of partially purified MDH

The column contained 1% Ampholine (LKB) pH range 3-10 and 5.0 mg of partially purified MDH protein. Electrolysis was carried out at 4°C for 60 hours at 300 volts and 0.3 mA. The column was emptied with a pump at a flow rate of 40 ml per hour and the effluent collected in 3.0 ml fractions. The protein containing tubes were combined to give 4 fractions. Each fraction was dialysed overnight against 2 vols of 0.01M Tris. HCl pH 8.0 to remove sucrose and Ampholine and then 0.01M Tris. HCl pH 8.0 containing 25% NaCl to reactivate MDH. Shading indicates the distribution of MDH activity.

○ o.d. 280 nm

● pH



Fraction No.

3.7 Characterisation of the purified MDH: The homogeneity of the purified MDH was investigated by gel-filtration and by polyacrylamide disc electrophoresis.

Gel-filtration on Sephadex: A Sephadex G100 column was prepared as described in paragraph 3.4. One ml of MDH containing about 1 mg of protein was applied to the top of the column and was then eluted with 0.01M Tris. HCl containing 25% NaCl. Figure 3.3 shows the distribution of protein and MDH activity in the eluate.

Polyacrylamide disc electrophoresis: Electrophoresis was carried out as described in paragraph 3.4. After staining for protein the gels revealed a prominent slow moving band and several other faster moving very much weaker bands. A diagram of a gel is shown in Figure 3.3.

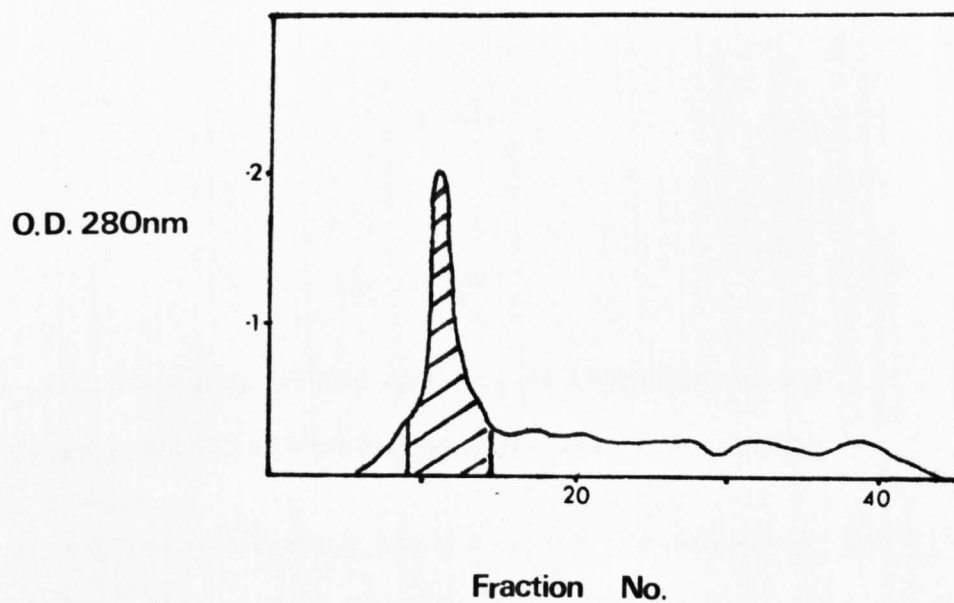
Figure 3.3

Characterisation of purified MDH by gel-filtration on Sephadex G100 and polyacrylamide disc electrophoresis.

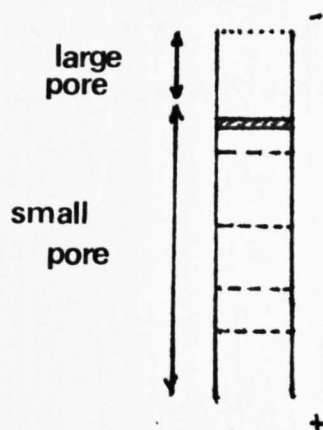
(a) Approximately 1.0 mg of MDH protein was applied to the top of a 50 x 1.5 cm column of G100 and eluted with 0.01M Tris. HCl pH 8.0, 25% NaCl buffer. Fractions of 3.0 ml were collected. Shading indicates the distribution of MDH activity.

(b) Gels contained a 3.5% acrylamide stacking gel and a 7% acrylamide running gel pH 9.5. Current was applied at 3 mA per tube for 10 minutes and 5 mA per tube for 90 minutes. Gels were stained for protein with Coomassie blue.

**a** Elution profile of G100



**b** Diagram of a gel



### 3.8 The effect of halophilic and pig heart MDH on the fluorescence of ANS

Weber and Young (1964) demonstrated that ANS binds strongly with bovine serum albumin (B.S.A.) and that its fluorescence emission maximum is shifted from 500 nm to 465 nm with a concomitant enhancement of intensity. However, many proteins do not intensify the fluorescence of ANS. For example, Stryer (1965) reported only small increases of fluorescence when 50 micromolar ANS was treated with 5 mg per ml solutions of pepsin,  $\alpha$  chymotrypsin, lysozyme or ribonuclease. In the light of these observations the effect on ANS fluorescence of halophilic and pig heart MDH (Sigma) was investigated.

The spectra were measured in the absence and presence of NaCl. Samples of halophilic and pig heart MDH were dialysed for 14 hours against two changes of 0.01M Tris. HCl pH 8.0 containing no added salt, or 4M NaCl. Aliquots of the resulting protein solutions containing 0.3 mg/ml protein were made 50 micromolar with a 5 mM stock solution of 1,8-ANS, ( $\text{NH}_4^+$  salt). The fluorescence emission spectra obtained when the samples were excited at 365 nm are shown in Figure 3.4.

Figure 3.4

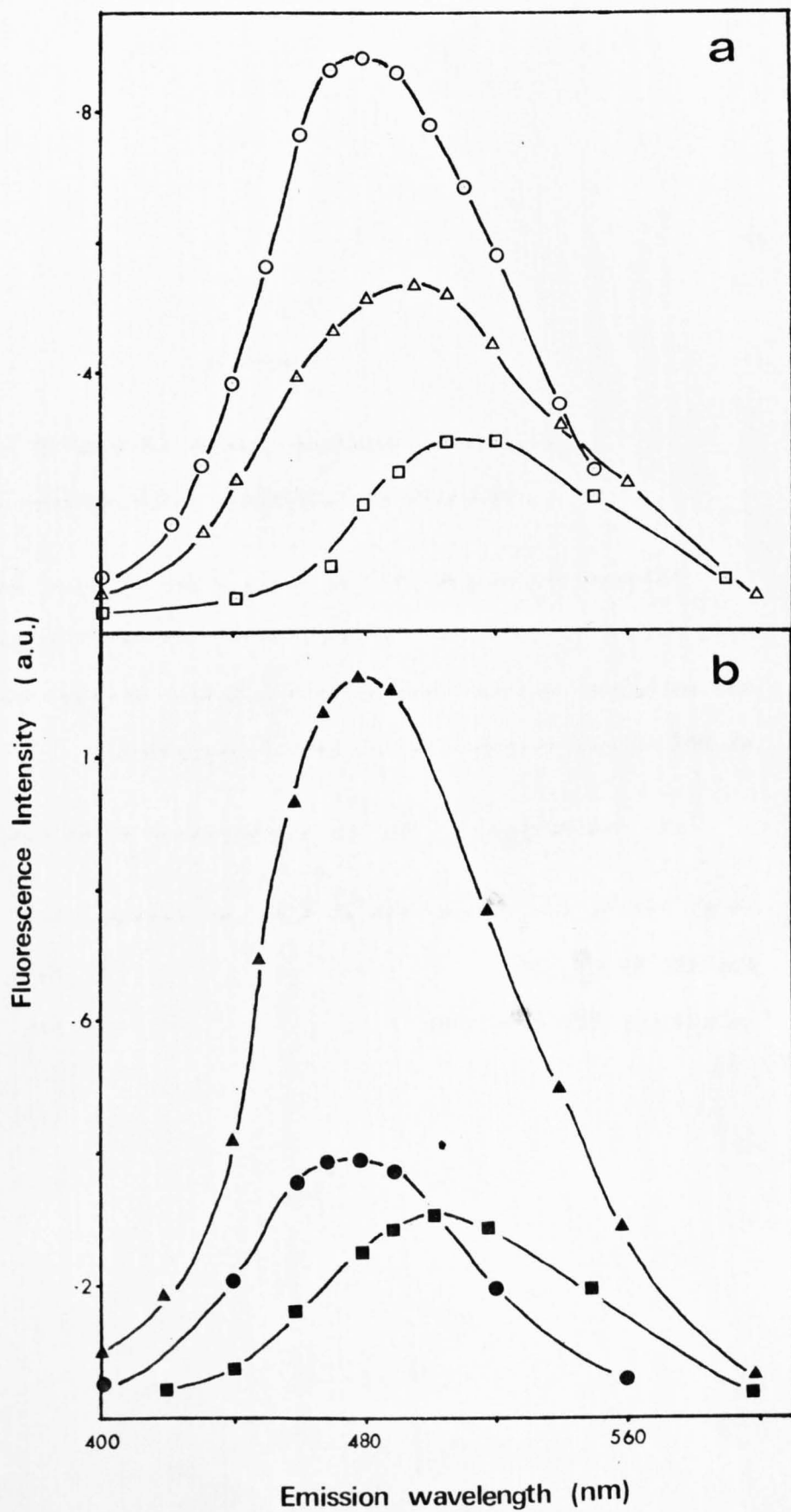
Fluorescence emission spectra of 1,8-ANS in the  
presence of halophilic and pig heart MDH

Aliquots of enzyme (0.3 mg protein per ml Tris. HCl buffer)  
were made 5  $\mu$ M with ANS. Spectra were measured with salt-treated  
and salt-free enzymes (see paragraph 3.8). Samples were excited  
at 365 nm and spectra read at room temperature.

(a) salt-free      (b) in the presence of 4M NaCl

50 $\mu$ M ANS in 0.01M Tris. HCl pH 8.0 (no protein)	(a) $\square$	(b) $\blacksquare$
Pig MDH 50 $\mu$ M ANS	(a) $\triangle$	(b) $\blacktriangle$
Halophilic MDH 50 $\mu$ M ANS	(a) $\circ$	(b) $\bullet$





### 3.9 The effect of NaCl concentration on the enhancement of ANS fluorescence by halophilic and mammalian MDH and B.S.A.

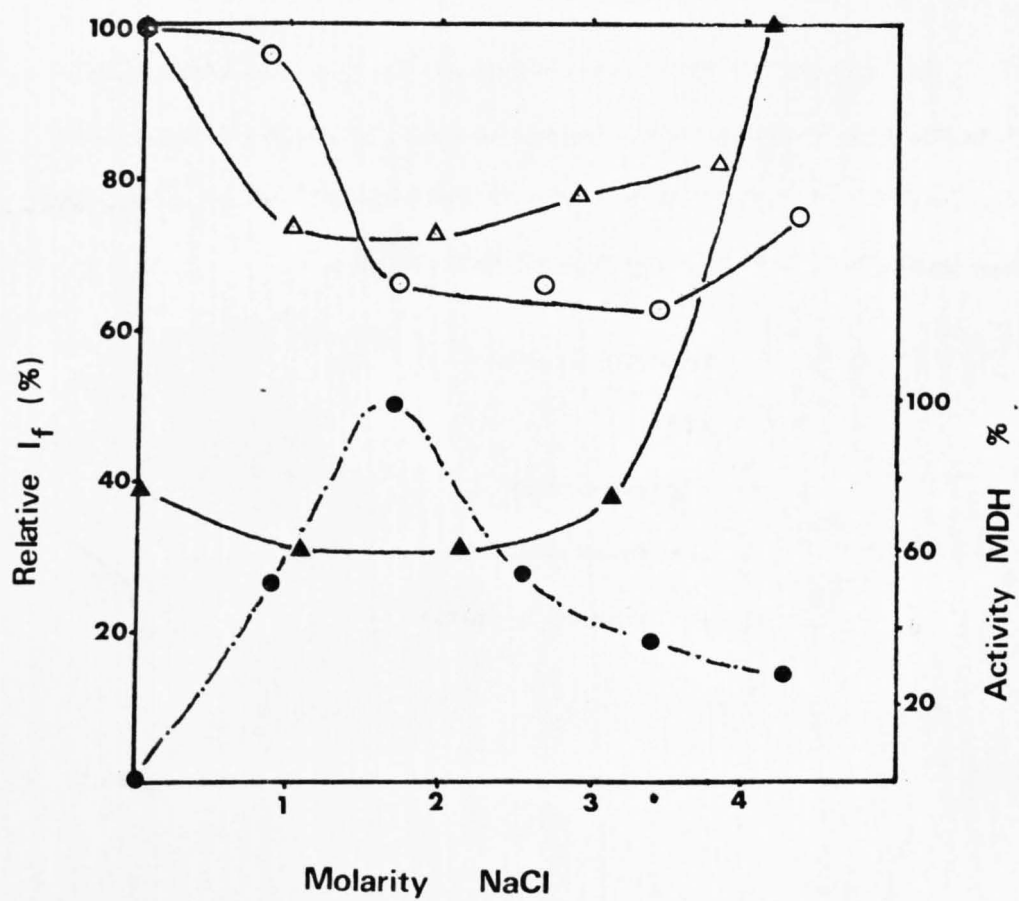
Samples of halophilic and pig heart MDH and BSA containing about 5 mg of protein in 0.005M Tris. HCl pH 8.0 were dialysed for 6 hours at 4°C against the same buffer containing concentrations of NaCl from zero to 4.3M. Aliquots were then taken containing 0.5 mg of protein, diluted to 2.0 ml with Tris. HCl buffer containing an appropriate concentration of NaCl and treated with 125 micromoles ANS. The fluorescence intensity of each sample was then measured at room temperature. Samples were excited at 365 nm with analyser settings as follows: halophilic MDH, 478 nm, pig MDH, 490 nm, and BSA, 470 nm. Figure 3.5 shows the variation of fluorescence intensity with salt concentration for each of the three proteins.

The specific activity of MDH in the halophilic enzyme samples was also determined.

Figure 3.5

The effect of NaCl concentration on the enhancement of 1,8-ANS fluorescence by halophilic and pig heart MDH and BSA. The relative specific activity of halophilic MDH is also shown. See paragraph 3.9 for experimental details.

- Halophilic MDH
- △ BSA
- ▲ Pig heart MDH
- Relative specific activity  
of halophilic MDH



3.10 The effect of the duration of exposure to NaCl on the ability of halophilic MDH to enhance the fluorescence of added ANS

If a slow conformational change is involved in the reactivation of halophilic MDH with salt then one might expect the ability of the enzyme to enhance ANS fluorescence to decrease on prolonged exposure to salt.

Sodium chloride was removed from purified halophilic MDH by dialysis for 24 hours at 4°C against several changes of 0.005M Tris. HCl pH 8.0. The salt-free enzyme was then reactivated at 4°C by dialysis against 0.005M Tris. HCl pH 8.0 containing 4.3M NaCl. At regular time intervals aliquots were taken containing 0.25 mg of protein. These were diluted to 2.0 ml with Tris. HCl-NaCl buffer and treated with 0.025 ml of 1,8-ANS (5mM). The fluorescence intensity of the solution was then measured. At the same time aliquots free of ANS were assayed for MDH activity. The results obtained are shown in Figure 3.6.

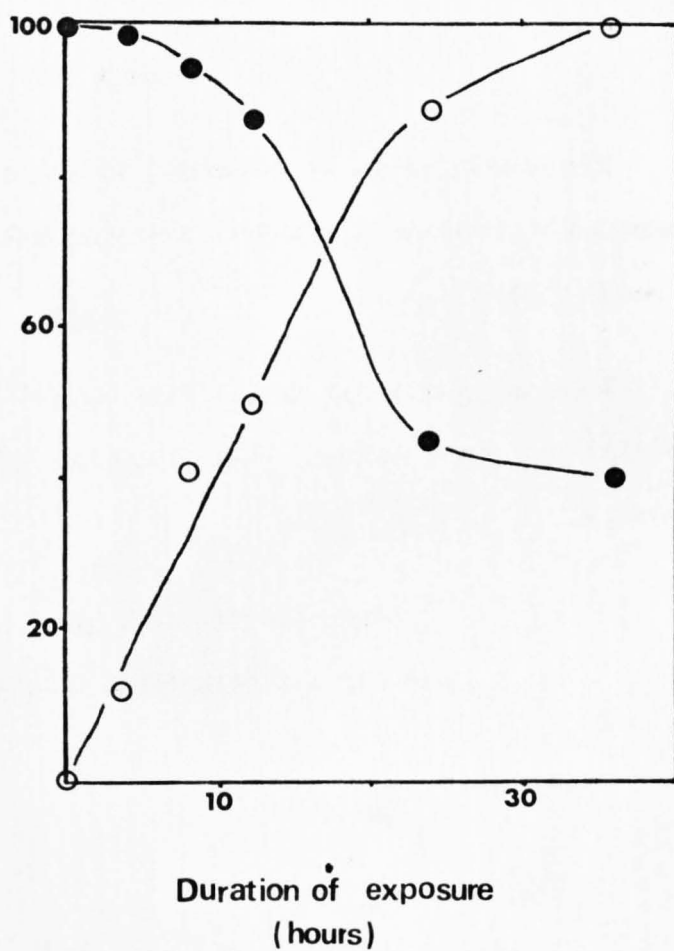
Figure 3.6

The reactivation of salt-free halophilic MDH by NaCl as measured by recoverable enzyme activity and ability to enhance 1,8-ANS fluorescence.

Data are presented as per cent change between observed initial and final values. See paragraph 3.10 for experimental details.

- Recoverable MDH activity
- Relative fluorescence intensity

Relative  $I_f$   
and  
MDH activity  
recovered  
(%)



### 3.11 The effect of preheated halophilic MDH on ANS fluorescence

The objects of this experiment were two-fold:

- (i) to investigate the effect of preheating the enzyme on its ability to enhance ANS fluorescence.
- (ii) to investigate the effect of NaCl on the sensitivity of the enzyme to heat treatment.

Purified MDH was dialysed overnight at 4°C against 0.01M Tris. HCl pH 8.0 to remove NaCl, or against Tris. HCl buffer containing 2.75M NaCl. Samples of the dialysed enzyme were heated at 50°, 60° and 65° for 10 minutes. Aliquots of heated enzyme containing 0.3 mg of protein were then diluted to 2.0 ml with Tris. HCl buffer (containing 2.75M NaCl in appropriate cases) and treated with 125 micromoles ANS. The fluorescence intensity (If) of each sample was then measured. Samples of heat treated enzyme were also assayed for MDH activity. Salt-free enzyme was reactivated by dialysis at 4°C for 30 hours against 25% NaCl in 0.005M Tris. HCl pH 8.0. The results obtained are summarised in Table 3.2.



Heat Treatment	Relative specific activity (%)		Relative fluorescence intensity (%)	
	2.75M NaCl	NaCl absent	2.75M NaCl	NaCl absent
Untreated	100	50	34	75
50°	59	52	74	100
60°	58	38	85	93
65°	42	47	80	95

Table 3.2 The stability of halophilic MDH to heat in the presence or absence of NaCl. (For experimental details see paragraph 3.11). "Untreated" enzyme was kept at 4°C. 100% represents the highest values obtained in either set of readings. Specific activity was measured as  $\mu$ moles NADH/min/mg protein.

### 3.12 The effect of spermine on the stability of halophilic MDH

Previously polyvalent cations such as spermine<sup>4+</sup> and Mg<sup>2+</sup> have been shown to produce partial activity and increased stability of salt-free halophilic enzymes (Hochstein and Dalton, 1968; Brown, 1969; Lanyi and Stevenson, 1970). Hochstein and Dalton claim to have obtained a fully active NaCl free NADH dehydrogenase preparation stabilised with 30mM spermine.

Multivalent ions participate in interionic interactions much more effectively than monovalent ions such as Na<sup>+</sup> (Derney and Moule, 1951). Thus they may stabilise halophilic enzymes in the absence of Na<sup>+</sup> by shielding polar moieties on the enzyme molecule.

Aliquots of crude enzyme (paragraph 3.2) containing about 10 mg protein were desalted by dialysis at 4°C for 24 hours against several changes of 0.01M Tris. HCl pH 8.0 containing zero, 5 or 50mM spermine tetrachloride. Details of the conditions and MDH activity upon removal of salt and reactivation are shown in Table 3.3, MDH activity as estimated by the assay procedure described in paragraph 2.6.

The results indicate that the presence of spermine in the absence of NaCl did not stabilise the enzyme sufficiently to retain activity but upon the return of salt almost full activity was recovered. The control in which the enzyme was kept in Tris-salt buffer throughout the dialyses also lost a proportion of activity.

Sample	MDH Activity (units/ml)	Desalting Buffer	MDH Activity after dialysis (units/ml)	Reactivating Buffer	MDH Activity after dialysis (units/ml)
Crude extract in Tris salt	5.1	Tris-salt	4.9	Tris-salt	3.0
Crude extract in Tris-salt	5.0	Tris	0	Tris-salt	1.2
Crude extract in Tris-salt + 50mM spermine tetrachloride	5.1	Tris + 50mM spermine tetrachloride	0.5	Tris-salt	4.3
Crude extract in Tris-salt + 5mM spermine tetrachloride	5.1	Tris + 5mM spermine tetrachloride	0	Tris-salt	4.5

Table 3.3 Effect of spermine tetrachloride on the activity of halophilic MDH upon the removal

of NaCl. Buffers : Tris-salt : 0.01M Tris HCl pH 8.0 + 25% NaCl

Tris : 0.01M Tris HCl pH 8.0

MDH activity : 1 unit is equivalent to 1  $\mu$  mole NAD per min

In the control in which NaCl was removed in the absence of spermine less than 50% of the original activity was recovered upon return of salt. Thus spermine appears to have stabilised the enzyme during dialysis to a greater extent than NaCl. The final recovery of activity was similar in both levels of spermine tested.

### Section III : Discussion

Many halophilic enzymes studied are irreversibly inactivated in the absence of high concentrations of neutral salts and this makes their purification and characterisation by conventional techniques difficult. However, certain enzymes, including MDH, can be reactivated after removal of salt by dialysis against high levels of NaCl, (Holmes and Halvorson, 1963). In these cases conventional separation techniques can be used in salt-free conditions, the enzyme being reactivated by treatment with salt. However, in the case of MDH a certain proportion of activity is lost by removal of salt, since at best, only 60% of the original activity can be reclaimed from the salt-free state. This means that the final yield of purified enzyme will suffer considerable reduction. However, the modified Holmes and Halvorson procedure employed gave a final preparation showing approximately an 850 fold increase of specific activity over the crude cell extract.

Characterisation of the purified product by gel-filtration and polyacrylamide disc electrophoresis revealed the presence of a predominant high M.W. constituent which was shown in the case of the G100 eluent to contain MDH activity. Isoelectric focusing was successful in purifying the enzyme preparation and showed MDH activity to be associated with protein with an isoelectric point (pI) within the range pH 5.0-6.0. Previously it has been suggested

that the proteins of halophilic organisms and particularly of the cell envelope are of an acidic character (Brown, 1965; Larsen, 1967). However, the value obtained for MDH is not unusually acidic since many proteins show lower values e.g. pepsin, 1.0; serum albumin 4.9;  $\alpha$ -casein 4.0 (Gordon-Young, 1963). However, recently Norberg and Hofsten (1970) have shown that an extra-cellular protease of H. salinarium strain 1M has an isoelectric point of pH 3.5 - 3.8. Possibly halophilic proteins exposed to the external environment under natural conditions tend to be of a more acidic character than intracellular proteins. MDH is certainly less halophilic than the cell envelope.

1,8-ANS interacts with both halophilic and pig heart MDH. This is accompanied by a blue shift in the emission of ANS and an enhancement of fluorescence intensity ( $I_f$ ), (Fig. 3.5). However, the conditions under which these effects are maximal for each enzyme are significantly different. In the salt-free state halophilic MDH causes a 30nm shift of the emission maximum ( $\lambda_{\max}$ ) and a large increase of  $I_f$ . In the presence of 4M NaCl the effect is smaller causing only a slight increase in  $I_f$  although  $\lambda_{\max}$  is shifted 28 nm. Pig MDH on the other hand shows greater effectiveness in enhancing ANS fluorescence in the presence of NaCl. The large increase in  $I_f$  is accompanied by a 20 nm shift of  $\lambda_{\max}$  compared to a 15 nm shift and about a 50% reduction of  $I_f$  in the salt-free state. ANS itself shows a shift of about 5 nm in the presence of

4M NaCl. Weber and Laurence (1954) showed that  $\lambda_{\max}$  decreases as the dielectric constant of the solvent is lowered. Increases in NaCl concentration up to 5M cause a linear decrease in dielectric constant (Harsted et al, 1948). Thus a small decrease in  $\lambda_{\max}$  of ANS would be expected in the presence of high levels of salt.

Variations of NaCl concentration effect the ability of BSA, pig and halophilic MDH to enhance ANS fluorescence, (Fig. 3.6). As the concentration of NaCl is increased from zero to about 1.75M  $I_f$  values observed with halophilic MDH decrease to a minimal value. This corresponds to the level of NaCl required for optimal MDH activity. A similar response is shown in Figure 3.7 where the observed change of  $I_f$  reflects the return of enzymic activity upon reactivation of the enzyme. Pig MDH, however, shows a low  $I_f$  values in lower levels of NaCl, but  $I_f$  increases markedly at high concentrations i.e. 3.0 - 4.0M. BSA, on the other hand, shows a response essentially similar to that of halophilic MDH.

Pig and halophilic MDH thus show opposite sensitivities to salt with respect to enzymic activity (Larsen, 1967) and ability to enhance fluorescence of ANS. Previously Holmes and Halvorson (1965b) demonstrated a similar response when they determined sedimentation coefficients of halophilic and pig MDH in the presence of various levels of NaCl. In high concentrations of NaCl pig MDH gave a low  $S_{20,w}$  value whilst halophilic MDH gave a

high value. In low salt these results were reversed. Hydrodynamic measurements have been used as a means of investigating shape properties of proteins in studies of the process of denaturation (Kauzmann, 1959). The low  $S_{20,w}$  values may be indicative of an unfolding of the protein molecules. This may result in the exposure of less-polar regions of the molecules leading to a greater interaction with ANS and an increase of fluorescence intensity.

Previously, Gally and Edelman (1965) have used ANS as a highly sensitive indicator of protein heat denaturation. Disruption of side-chain interactions responsible for protein tertiary structure by surface forces, solvent changes or thermal changes may result in the exposure of hydrophobic groups which were originally in the interior of the molecule. Since ANS interacts more strongly with hydrophobic sites on proteins (Stryer, 1965) a concomitant increase of fluorescence intensity may result on denaturation.

In agreement with the result obtained by Holmes and Halvorson (1965b) halophilic MDH was found to be more sensitive to heat denaturation in the presence of NaCl than in the salt-free form, (Table 3.2). At 65°C the native enzyme lost more than 50% of its activity, whereas the salt-free enzyme was stable. Since the latter samples were dialysed against salt-free buffer prior to heat treatment only about 50% of the original activity would be expected on reactivation. The salt-free enzyme gave highest  $I_f$



values, although its ability to enhance fluorescence increased by only 30% after heat treatment at 50°C. However, the greater sensitivity of the native enzyme to heat was also reflected by an increase in fluorescence enhancement of over two-fold after heating at 50°C. Preheating the enzyme thus causes a substantial increase in  $I_f$  even in the NaCl-free form possibly indicating that denaturation is not complete upon the removal of salt.

The observation that the enzyme is more heat labile in the presence of  $\text{Na}^+$  is rather unexpected since in a halophilic enzyme one might anticipate that  $\text{Na}^+$  would tend to act on a stabiliser. Indeed Hubbard and Miller (1970) reported that  $\text{Na}^+$  stabilised halophilic isocitrate dehydrogenase from H. cutirubrum against inactivation by alkylating or oxidising agents which attack sulphhydryl groups e.g. iodoacetamide, N-ethyl maleimide. Thus it is possible that the overall stability of the MDH does not depend solely upon interionic interactions with NaCl.

## Chapter IV : Isolation and Characterisation of the Cell Membrane

### Introduction

For many years it was thought that a thick cell wall of the type present in Gram negative bacteria was absent from Halobacteria, the limiting structure of the cells being a single triple layered "unit membrane" like structure. Recently with improved techniques of electron microscopy the nature of the cell envelope has been shown to be more complex, the cell membrane being overlaid with a proteinaceous layer, 75-150 Å thick, (Stoeckenius and Rowen, 1967; Steensland and Larsen 1969). The latter authors also demonstrated a  $Mg^{2+}$  stabilised amino sugar layer sandwiched between the outer  $Na^+$  stabilised protein coat and the inner cell membrane. This layer may correspond with the mucopolysaccharide found in other Gram negative organisms (Salton, 1964), although components characteristic of this layer, e.g. muramic acid, have not been detected. (Kushner and Onishi, 1968).

This chapter describes the isolation and purification of the cell membranes of H. salinarium strain 1 and 1M. Crude membrane preparations were purified by gel-filtration on Agarose. The fractions released were characterised by electron microscopy, sucrose density gradient centrifugation and polyacrylamide gel electrophoresis (PGE). The effect of urea on purified membrane was also investigated, the products of disaggregation being

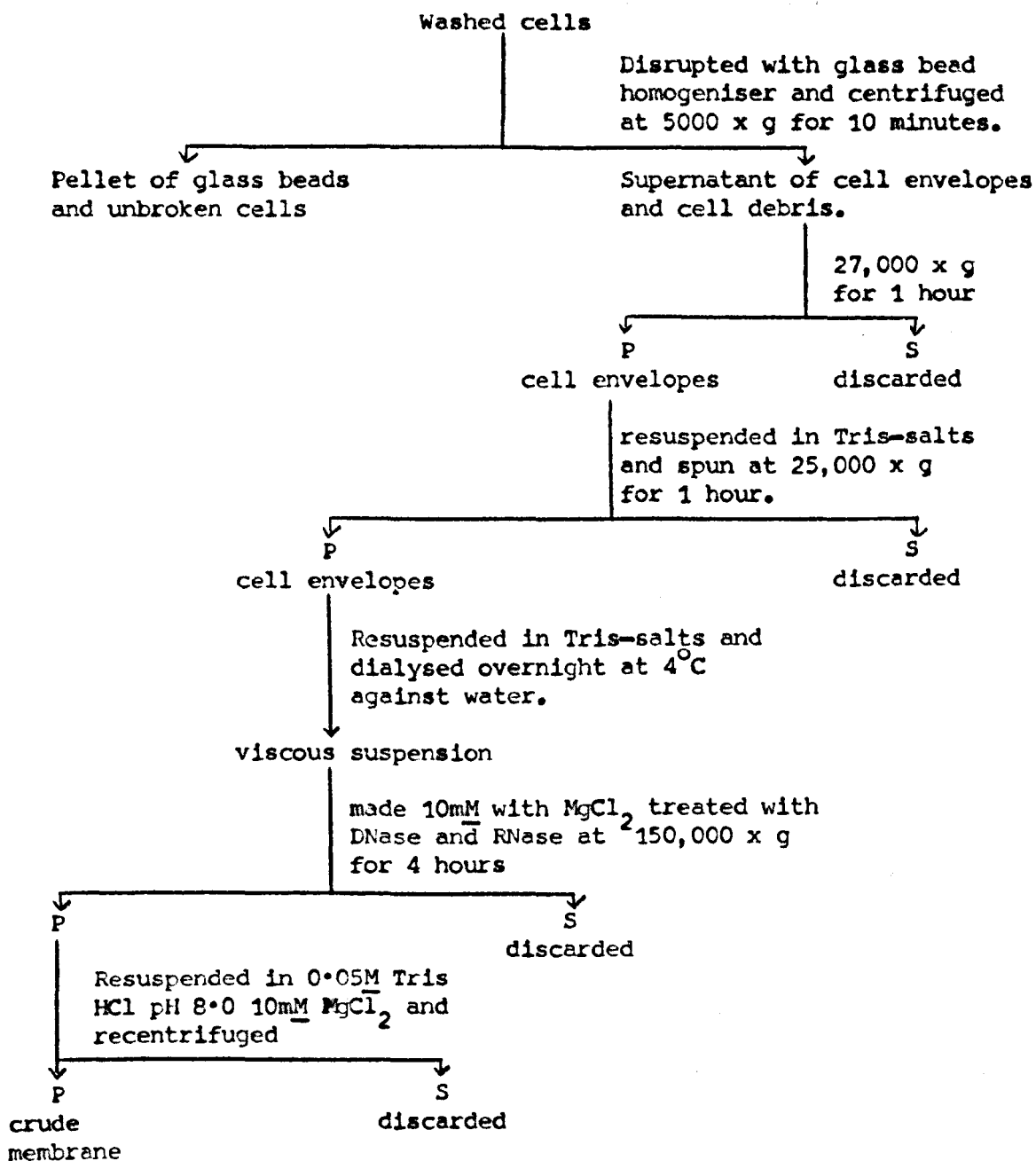
identified by PGE, amino acid analysis and gel-filtration. A report of part of this work awaits publication (Brown, Bellingham and Stevenson, 1971).

## Section I : Methods

4.1 Isolation of cell envelopes: The method used was based upon that described by Steensland and Larsen (1969). A flow chart summarising the preparation of cell envelopes and crude membrane is shown on page 4.3. Washed cells were disrupted using a Braun glass bead homogeniser and the resulting cell envelopes isolated as described in detail in paragraph 2.6.

4.2 Isolation of crude membrane from cell envelopes: A pellet of washed cell envelopes was suspended in about 5 volumes of cold Tris-salts and dialysed at 4°C overnight against 51 of distilled water. This treatment caused the release of nucleic acids and the resulting highly viscous dialysate was treated with 0.1 µg of DNase and RNase in the presence of 10mM MgCl<sub>2</sub>. Incubation at room temperature with gentle stirring for about one hour gave a suspension which was suitable for centrifuging at 150,000 xg for 4 hours at 4°C in a Beckman model L-2 ultracentrifuge. The pellet of membrane obtained was resuspended in 10mM MgCl<sub>2</sub> and respun. Samples of washed membrane were stored at -20°C in tubes covered with foil.

Summary of cell membrane preparation



4.3 Purification of the crude membrane by gel-filtration: Frozen crude membrane was thawed overnight in the presence of 0.05M Tris HCl pH 8.0 at 4°C and resuspended by gentle use of a Potter-Elvehjem teflon homogeniser. A sample of this solution containing about 150 mg of protein was applied to a 30 x 5 cm Agarose column (Bio-Rad, A50) pre-equilibrated with 0.05M Tris HCl pH 8.0 containing 0.1M NaCl. The column was fitted with adaptors for upward flow of buffer which was pumped with a Watson and Marlow peristaltic pump at a flow rate of 15 ml per hour. The absorbancy of the effluent at 254 nm was monitored with an L.K.B. Uvicord and collected in 5.0 ml fractions with an L.K.B. "UltroRac" fraction collector on automatic time change. The apparatus was installed in a cold room at 4°C.

The fractions containing individual protein peaks were combined and concentrated using an Amicon Ultrafilter fitted with a UM-2 membrane which only allowed the passage of material of molecular weight less than 1,000.

4.4 Centrifugation of membranes on sucrose density gradients: 30 ml of linear sucrose gradient of 5-70% sucrose in 0.01M Tris HCl pH 8.0 were prepared using an L.K.B. gradient mixer. Samples of membrane containing about 20 mg of protein dissolved in 1-2 ml 0.01M Tris HCl pH 8.0 were carefully layered on to the top of the gradients. The tubes were spun at 60,000 xg for 2½ hours using an SW 25.1 rotor in a Beckman L2 ultracentrifuge at 4°C.

The contents of the tubes were analysed by piercing the tubes with an M.S.E. tube piercer and collecting 0.5 ml fractions with the aid of a peristaltic pump and a fraction collector. The fractions were diluted to 1.0 ml with 0.01M Tris HCl pH 8.0 and their absorbancy at 280 nm and 254 nm measured using a Zeiss PMQII spectrophotometer.

4.5 Polyacrylamide disc electrophoresis: The method used was based upon that described by Davis (1964). Polyacrylamide gels were prepared consisting of 0.15 ml of 3.5% acrylamide stacking gel containing pH 6.7 Tris HCl and 0.9 ml of 7% acrylamide running gel containing pH 8.9 Tris HCl. The tubes were set up in a Shandon electrophoresis vessel containing 0.03M sodium borate pH 8.9 in the electrode resevoirs.

The density of solutions for electrophoresis was increased by the addition of 1 drop of 70% sucrose and aliquots of 0.1-0.2 ml containing 100-200 µg of protein were carefully layered on to the top of the gels. The anode from the power supply was connected to the lower resevoir electrode. The current was switched on for 10 minutes at 3 ma per tube and 90 minutes at 5 ma per tube and 200 volts. At the end of the run the gels were stained for protein using Coomassie Blue according to the method of Chrambach et al (1967), or nucleotides with methylene blue (Dahlberg, Dingman and Peacock, 1969). Lipo-proteins were detected by pre-staining with Sudan Black B, (Narayan et al, 1966).

Preparative P.G.E. was carried out using Shandon apparatus. A 10 cm column of gel was prepared containing 7% acrylamide, 6M urea and 0.05 M pH 8.9. Tris HCl. The electrode resevoirs contained 0.03 M sodium borate pH 8.9. The bottom surface of the gel column was eluted with 0.05 M Tris HCl at a flow rate of 20 ml per hour, and the effluent was monitored at 254 nm with a Uvicord and collected in 3.0 ml fractions. At the start of the run power was adjusted to 30 mA and 160 volts. The front band was obtained after 3 hours. No further peaks were recovered.

4.6 Gel filtration on Sephadex: Quantities of Sephadex (Pharmacia) G200, G100 and G25 were preswollen in 0.01M Tris HCl pH 8.0 containing 0.1M NaCl. Slurries of the swollen gels were poured and equilibrated to give 55 x 1.5 cm columns. Protein solutions containing 5-50 mg of protein were applied to the top of the columns and buffer was pumped at 15 ml per hour using a peristaltic pump. The optical density of the effluent was monitored at 254 nm using an L.K.B. Uvicord and recorder and fractions of 2 ml were collected using an "UltroRac" collector.

4.7 Estimation of cytochromes: Cytochromes were estimated by measuring difference spectra using sodium dithionite as reducing agent. The cytochrome content was expressed in arbitrary units (A.u. per mg protein) defined as  $A_{427 \text{ nm}} - A_{480 \text{ nm}}$  (Bednar, 1965). Spectra were measured using a Unicam SP 800 spectrophotometer.

4.8 Estimation of carotenoid: Samples were made 15% (w/v) with

NaCl and extracted with 2 volumes of acetone. Acetone was removed by aspiration under nitrogen and the residue was dissolved in ether. The absorption at 494 nm was measured using a Zeiss PMQII spectrophotometer and calculations were based on an  $E_{1\text{cm}}^{1\%} = 2500$  (Liggen Jensen, 1962).

4.9 Nucleic acid determination: Nucleic acid and nucleotides were estimated by the orcinol reaction (Schneider, 1957).

4.10 Amino acid analysis: Protein or lipoprotein samples to be analysed were dialysed for 48 hours at 4°C against several changes of distilled water. Duplicate aliquots containing 1-2 mg of protein were then placed in Pyrex hydrolysis tubes and treated with 0.25 ml of micro-analytical grade concentrated HCl (B.D.H.) and 9.25 ml of 0.001M norleucine in 0.01M HCl. The norleucine was added as an internal standard to determine the overall recovery of amino acids after hydrolysis and chromatography. (Walsh and Brown, 1962). The tubes were then frozen in liquid nitrogen and sealed under vacuum.

Hydrolysis was carried out by placing the tubes in a heating block in an oven at 110°C for 22 hours. The tubes were then cooled and HCl removed under vacuum in the presence of solid KOH and concentrated H<sub>2</sub>SO<sub>4</sub> (Beckman Amino Acid Analyser Manual, A-1M-3, 1965). The final residue was dissolved in 5 ml of 0.2M sodium citrate, pH 2.2 and stored at 4°C (Moore and Stein, 1954).



The analysis of hydrolysates was carried out using a Beckman model 120C amino acid analyser set up as described in the manufacturers' instruction manual. The analysis of basic amino acids was performed using PA35 resin in place of the AA27 resin supplied with the instrument. Previously it had been found that much better resolution of the basic amino acids could be obtained using a 1 cm column of PA35 resin (Brown et al, 1971). The overall recovery of amino acids as estimated by the final concentration of norleucine was usually in the range 90-95%. Corrections of the analysis were made for expected losses of serine (10%), threonine (5%), Tyrosine (5%) and glutamic acid (5%) as determined by Hirs et al (1956). Any methionine sulfoxide and allo-isoleucine detected were included in the final values of methionine and isoleucine respectively. Cysteine was calculated as the sum of cysteine, cystine and cysteic acid. Ammonia values were corrected for the expected decomposition of amino acids during the hydrolysis.

#### 4.11 Preparation of membrane material for electron microscopy:

The method of fixation used was that described by Steensland and Larsen (1969). One volume of membrane suspension was treated with one volume of half strength Glauert and Thornley (1966) fixative, i.e. 2.5% glutaraldehyde in 0.1M sodium cacodylate HCl pH 7.1, 0.01M  $\text{CaCl}_2$ , and incubated at 4°C for 1 hour. The material was then centrifuged at 27,000 g for 30 minutes and the pellet washed 3 times by resuspension in 0.1M cacodylate buffer. The pellet was

then suspended and left overnight at 4° in osmium tetroxide fixative (no tryptone), (Kellenberger, Ryter and Séchaud, 1958). The next morning the pellets were allowed to warm up to room temperature and were washed with 6 volumes of Kellenberger acetate - veronal buffer and then washed for 30 minutes with Kellenberger uranyl acetate solution. The preparations were then dehydrated in a series of ethanol - water solutions finally being taken up in acetone and embedded in Vestopal 310 (Chemische Werke Huls A - G, W. Germany) using Trigonox CM-50 (Novadel, Kent) and cobalt naphthenate as initiator and accelerator respectively.

The embedded material was sectioned, stained with 2% uranyl acetate and 0.002% lead citrate and examined with a Siemens Elmiskop at the Technical Institute of Norway by Mrs Asbjorg Flo.

## Section II : Results

4.12 Purification of crude membranes: Samples of crude membrane from H. salinarium strain 1 or 1M gave similar results when they were eluted from an Agarose A50m column. An example of an elution pattern obtained is shown in Figure 4.1. About 65% of the total membrane protein applied to the column was eluted in the void volume. The exclusion limit of the gel quoted by the manufacturers indicates that this material has a molecular weight greater than  $50 \times 10^6$  (see Figure 4.2). This fraction also

Figure 4.1

Gel-filtration of crude membrane on Agarose A50m

A sample of crude membrane containing about 150 mg of protein was applied to a 30 x 5 cm column equilibrated with an upward flow of 0.05M Tris HCl pH 8.0, 0.1M NaCl buffer at 4°C. The column was eluted with the same buffer and fractions of 5.0 ml were collected. Shading indicates the distribution of cytochrome and carotenoid.

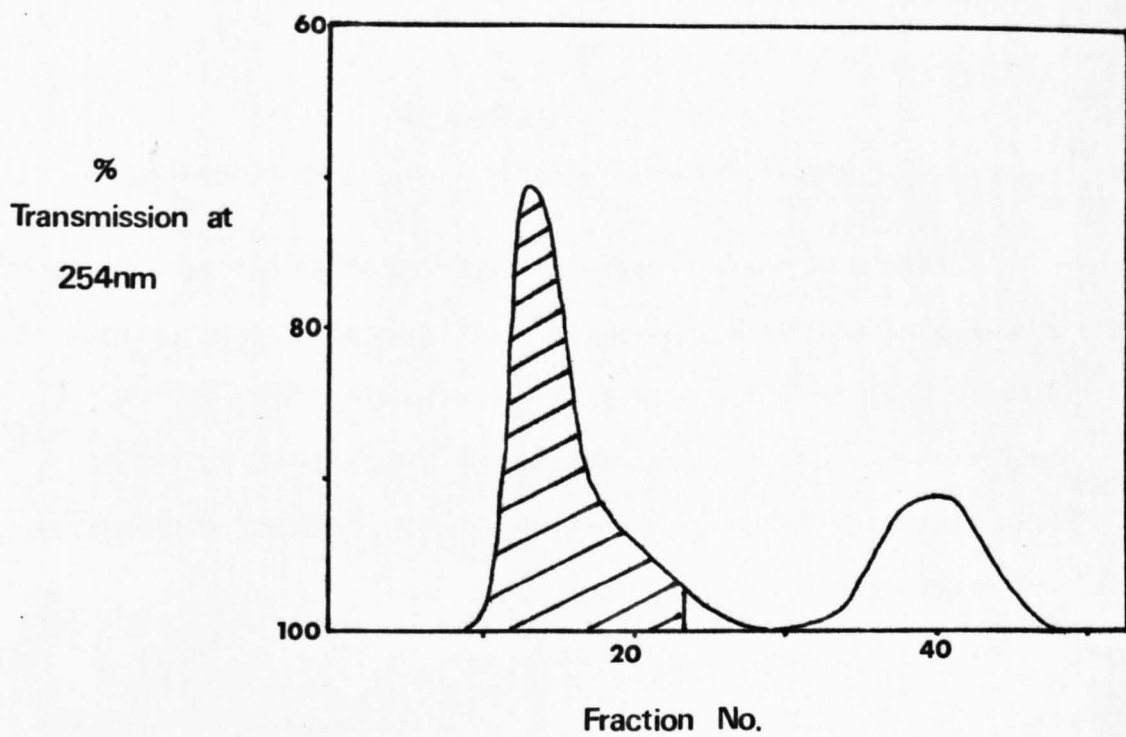
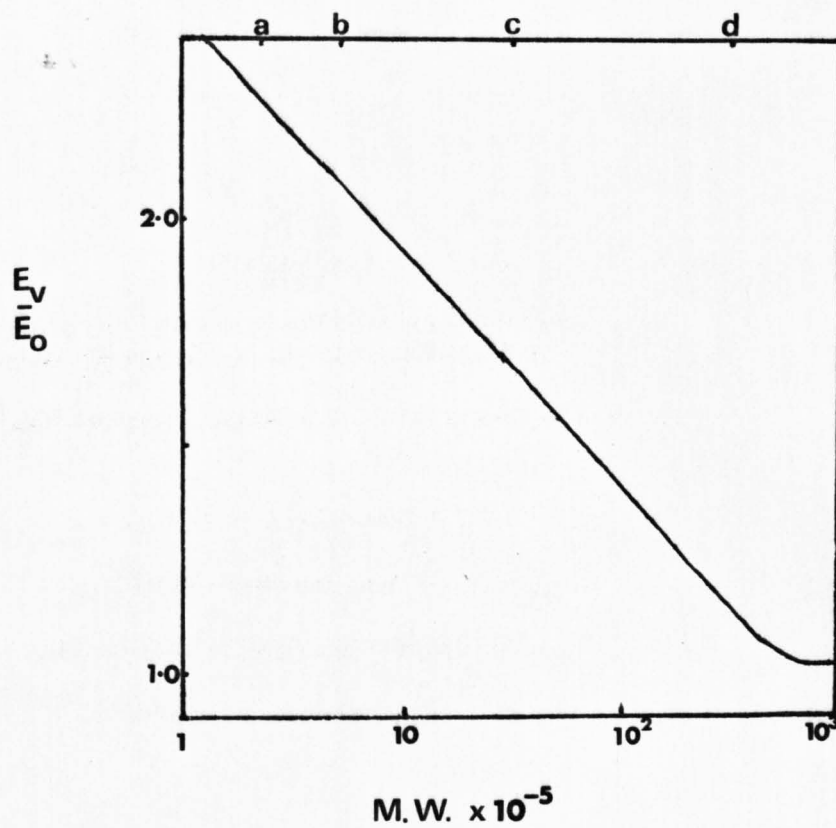


Figure 4.2

Relationship between molecular weight  
and elution volume on Bio-Gel A-50m

(Reproduced from Bio-Rad leaflet BG-5E)

- (a) Human gamma globulin
- (b) Bovine thyroglobulin
- (c) Southern bean mosaic virus
- (d) Tobacco mosaic virus



contained all of the cytochrome, and carotenoid in the case of strain 1, that was present in the crude membrane. The remaining 35% of sample protein was retained on the gel and came off in the bed volume. This latter fraction also contained ribonucleotide which was present in the crude membrane to about 2% of the dry weight. When this nucleotide-protein complex was concentrated using a "Diaflo" ultra-filter with an exclusion limit of 1000, the protein was retained by the filter membrane free of nucleotide. Gel-filtration of this concentrated protein on a Sephadex G25 column indicated a molecular weight of about 2000.

The proportion of nucleotide-protein complex that was released from the crude membrane by gel-filtration was affected by the presence of  $Mg^{2+}$  (Brown, Bellingham and Stevenson, 1971). For example, if  $1mM MgCl_2$  was included in the Tris HCl eluate only 25% of the total membrane protein applied was eluted in the bed volume of the column. The purification of crude membrane was routinely carried out using magnesium free buffer.

The results of polyacrylamide disc electrophoresis of purified membrane and nucleotide-protein complex are illustrated in Figure 4.3. Similar results were obtained for strain 1 or 1M. The nucleotide-protein complex also gave the same distribution of bands irrespective of whether the gel-filtration on A50m was carried out in the presence or absence of magnesium. Of the five bands revealed by protein

Figure 4.3

Polyacrylamide gel disc electrophoresis of purified membrane and nucleotide-protein complex derived from crude membrane by gel-filtration on Agarose A50m.

(A) purified membrane

(B) nucleotide-protein complex

Method as described in paragraph 4.5. Gels were stained for protein, lipid or nucleotide.

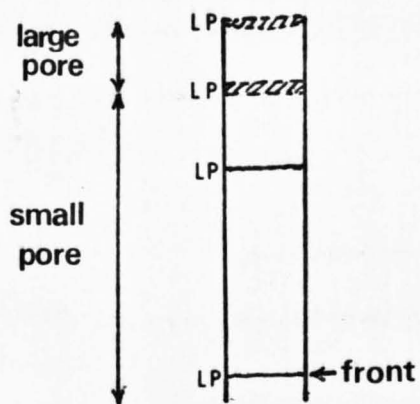
Key: P = stains for protein

L = " " lipid

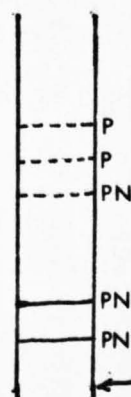
N = " " nucleotide



**A**



**B**



stain, none stained for lipid and only three for nucleotide. None of these bands were present in the purified membrane gel. As expected from the very high molecular weight of the purified membrane material, most of the sample remained at the origin, or stopped at the top of the small pore running gel. However, two bands were always detected, whether the membrane was purified with or without  $Mg^{2+}$  in the buffer - a slow moving band and a fast band which moved at the migration front. Both of these bands stained weakly for lipid and strongly for protein and neither were present in the nucleotide-protein complex gels.

Amino acid analyses of purified membrane and the nucleotide-protein complex are shown in Table 4.2, paragraph 4.15. The nucleotide-protein complex contains higher proportions of the polar amino acids and rather less of the apolar.

#### 4.13 Physical characterisation of the crude and purified membranes

The physical nature of crude and purified membranes from H. salinarium strain 1 and 1M were investigated by electron microscopy and sucrose gradient centrifugation.

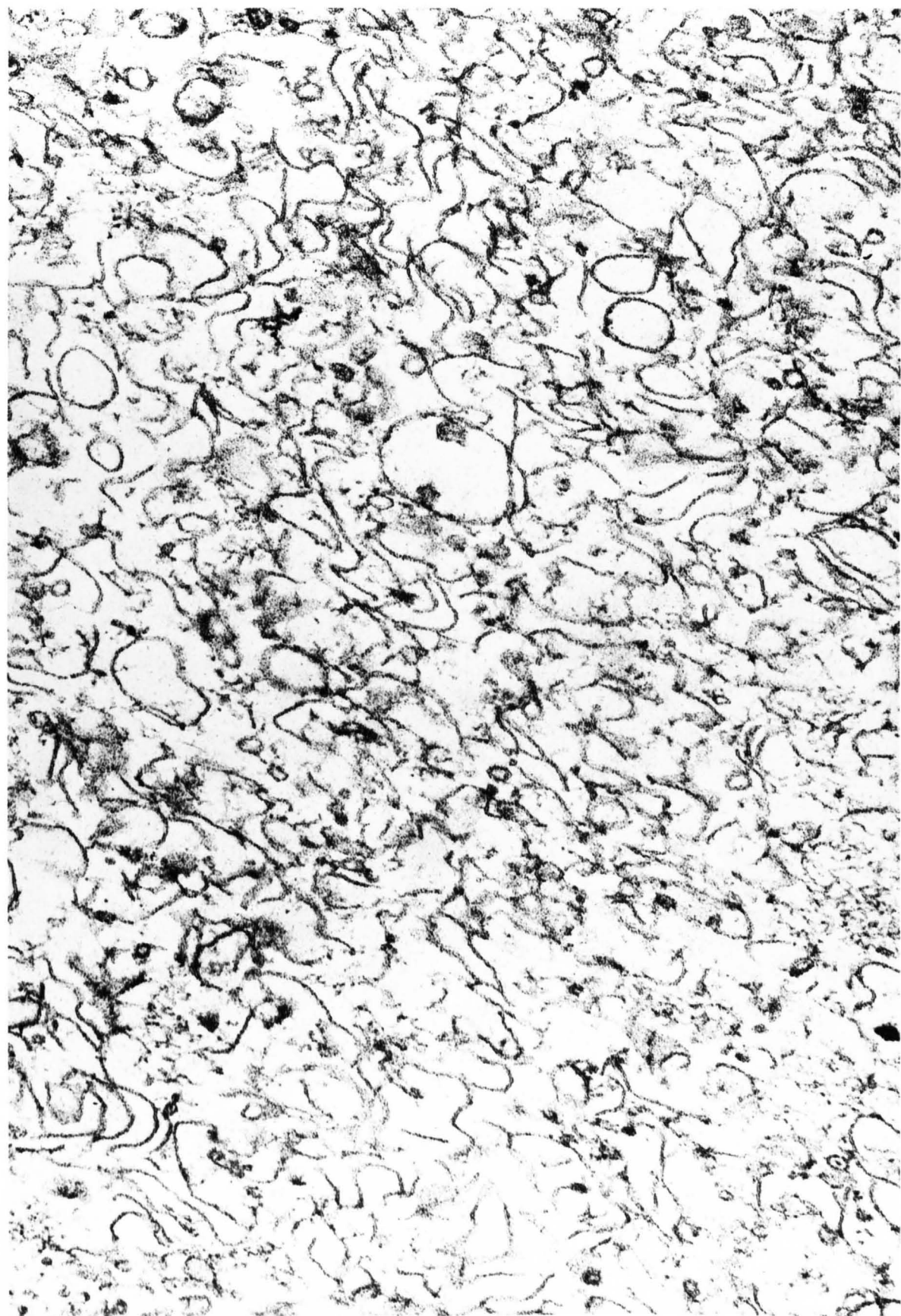
Electron microscopy: Samples of crude and purified membrane were fixed and stained as described in Section I of this chapter. The electron micrographs obtained are shown in Figure 4.4. All preparations show a considerable proportion of material with the typical appearance of triple layered unit membrane structures. Many vesicles are apparent, especially in the crude membrane

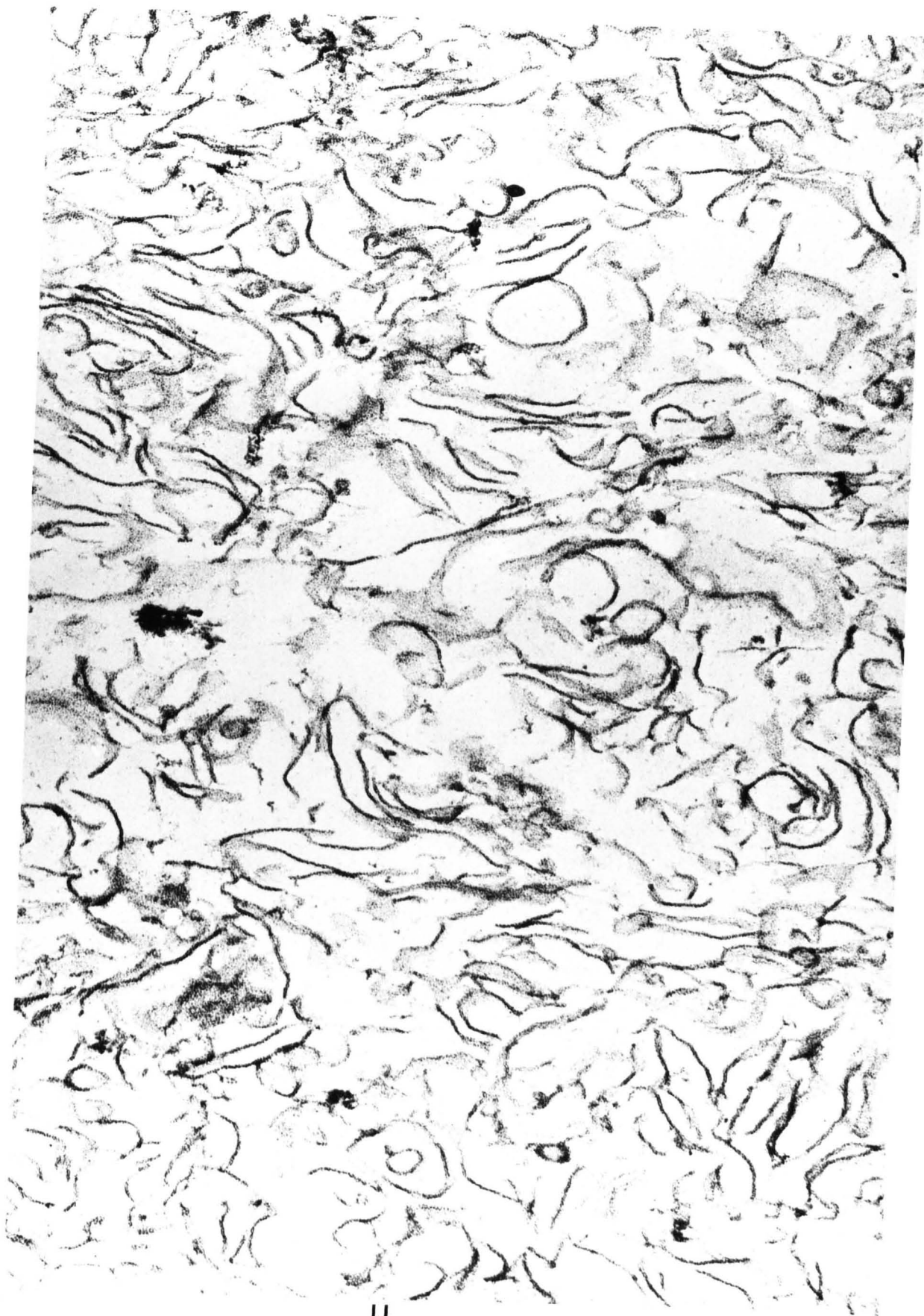
Figure 4.4

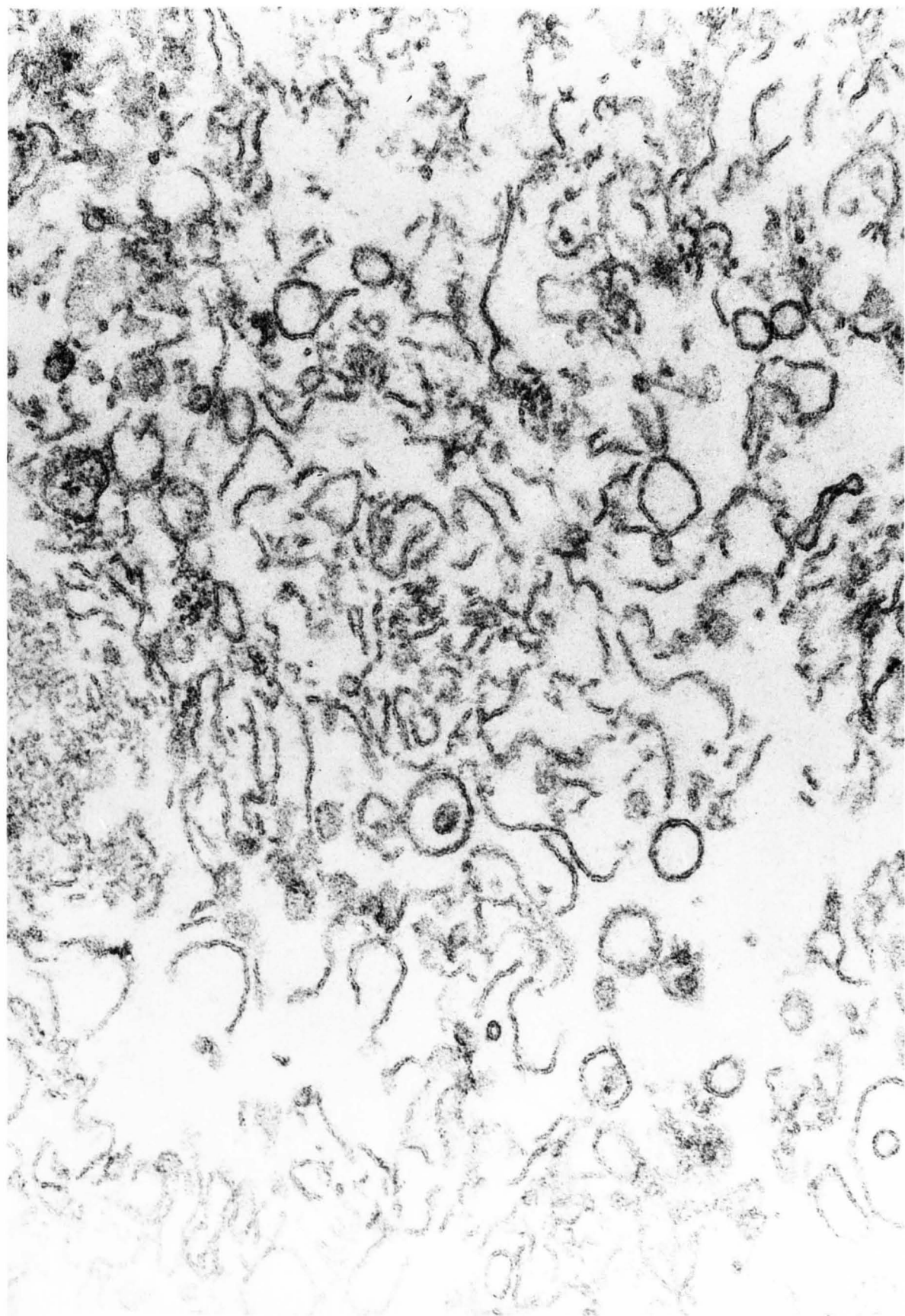
Electronmicrographs of H. salinarium strain 1 and 1M cell membranes  
(magnification x 70,000)

Details of the fixing and staining technique used are given in  
paragraph 4.10.

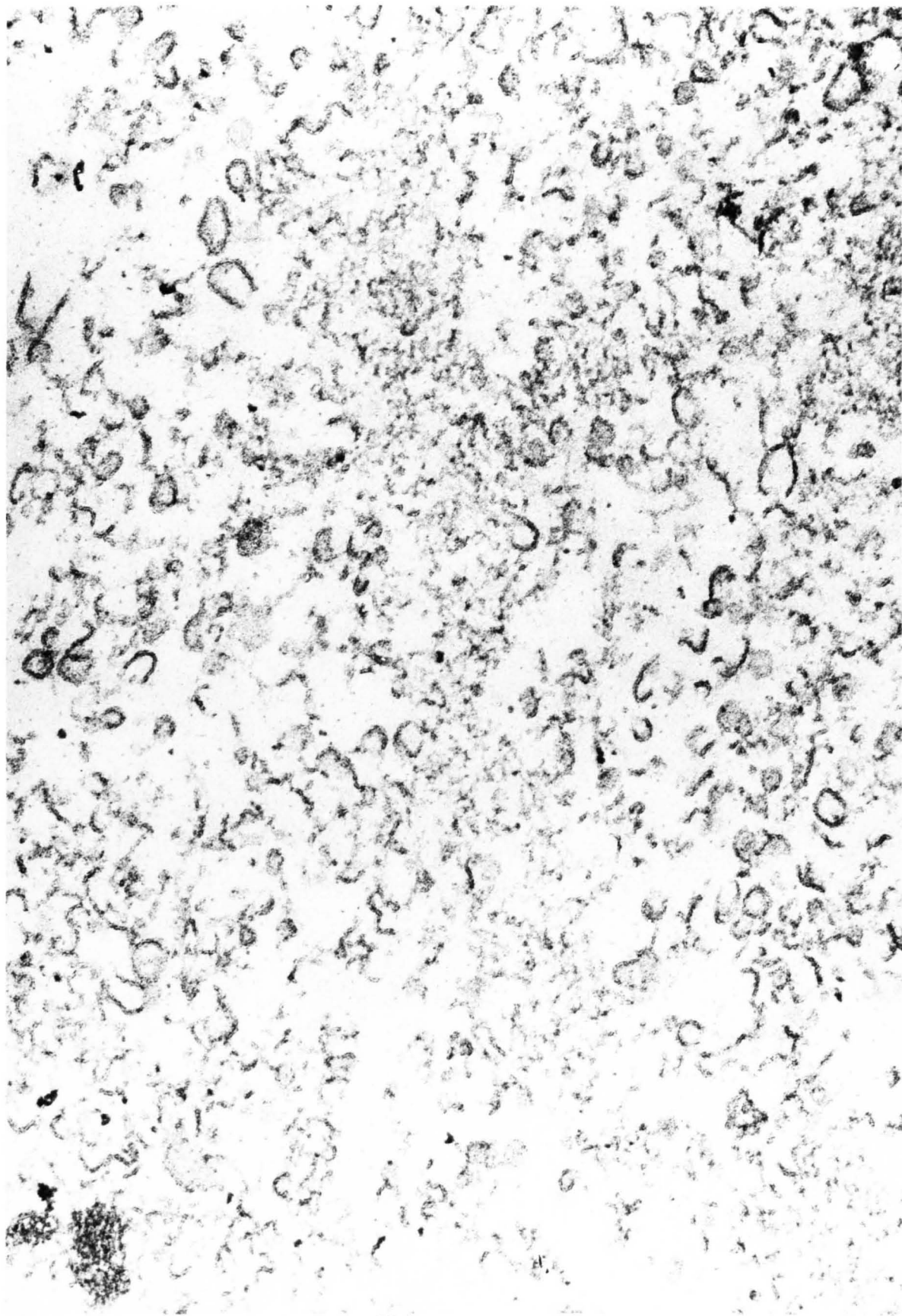
- Plate    I : Crude membrane strain 1  
          II : Purified membrane strain 1  
          III : Crude membrane strain 1M  
          IV : Purified membrane strain 1M











samples, some of which contain entrapped material. The purified membrane preparations show considerable disruption of the vesicles into fragments some of which are clearly triple layered, whilst the remainder resemble the non-membranous material seen in crude membrane preparations.



Sucrose gradient centrifugation: Membrane samples from strain 1 and 1M gave similar results when centrifuged on sucrose gradients. The results obtained using crude and purified membrane from strain 1 are illustrated in Figure 4.5. In the case of crude membrane 60-70% of the total protein in the sample was accounted for by the heavier fraction. This band was red and contained cytochrome. The rest of the protein remained at the top of the gradient. The optical density at 254 nm of this light fraction was significantly higher than at 280 nm indicating the presence of nucleotides as well as proteins. When the light fraction was concentrated, washed with Tris HCl buffer on an ultrafilter and analysed by disc electrophoresis a gel was obtained as illustrated in Figure 4.6. The pattern of bands was similar to that produced by the nucleotide-protein complex released by crude membrane during purification by Agarose gel-filtration.

Purified membrane also gave two bands in the sucrose gradient. The dense band contained carotenoid and cytochrome and accounted for about 80% of the sample protein. The light band was colourless, apparently free of cytochrome and represented 15-20% of the sample protein. This band was unlike the light fraction from crude membrane in that it penetrated the gradient and did not have a significantly different optical density at 254 nm and 280 nm.

The heavy band produced by purified membrane travelled at a

Figure 4.5

Centrifugation of crude and purified membrane  
for strain 1 on sucrose density gradients

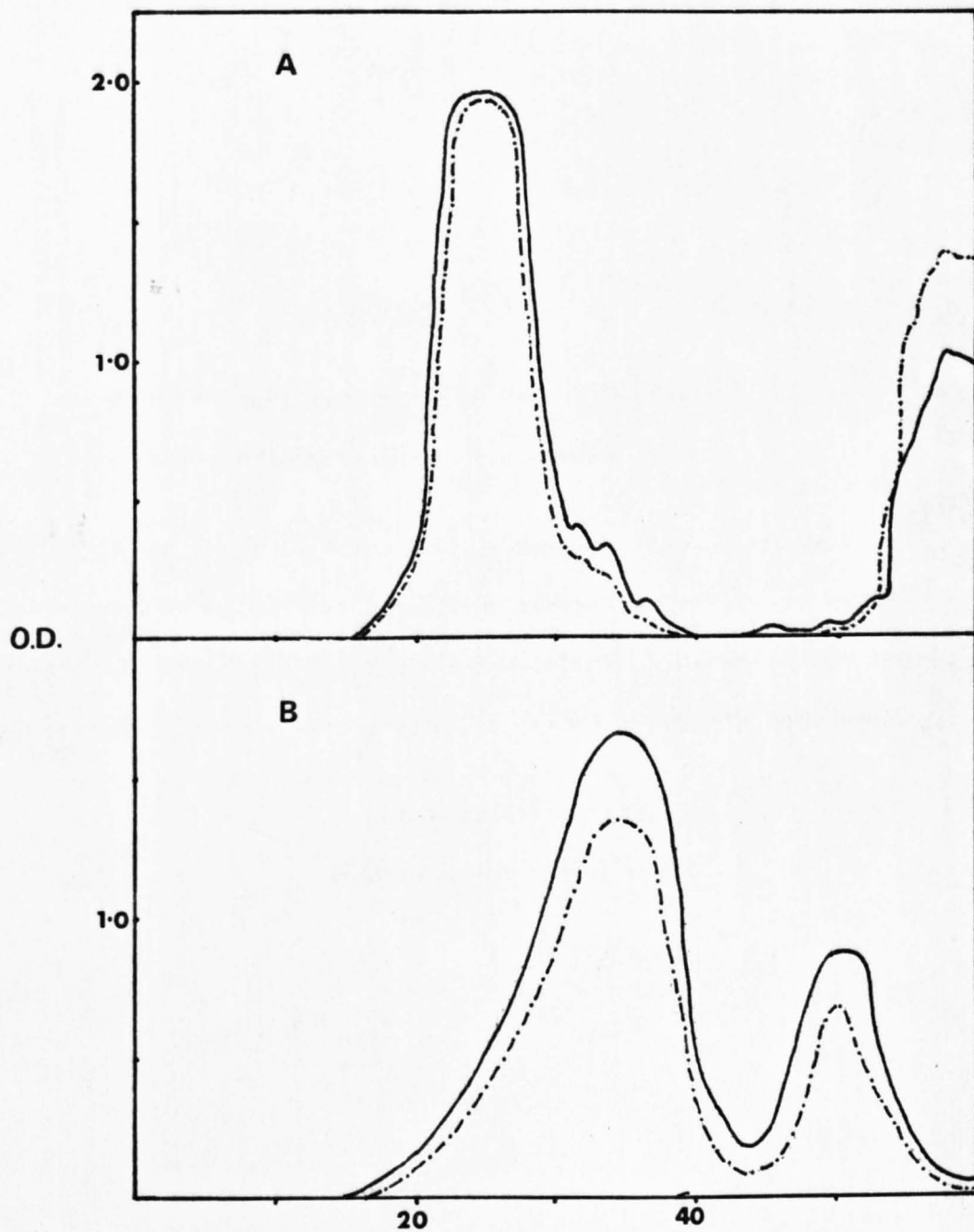
Samples of membrane containing about 20 mg of protein were layered on to linear sucrose gradients containing 5-70% sucrose and spun for  $2\frac{1}{2}$  hours at  $60,000 \times g$  at  $4^{\circ}\text{C}$ . The ends of the tubes were pierced and 0.5 ml fractions collected (see paragraph 4.4 for details).

(A) Crude membrane

(B) Purified membrane

o.d. 280 nm ———

o.d. 260 nm - - - - -



Fraction No.

Figure 4.6

Diagrams of gels from polyacrylamide disc gel electrophoresis of fractions obtained by sucrose density gradient centrifugation of crude and purified membrane from strain 1.

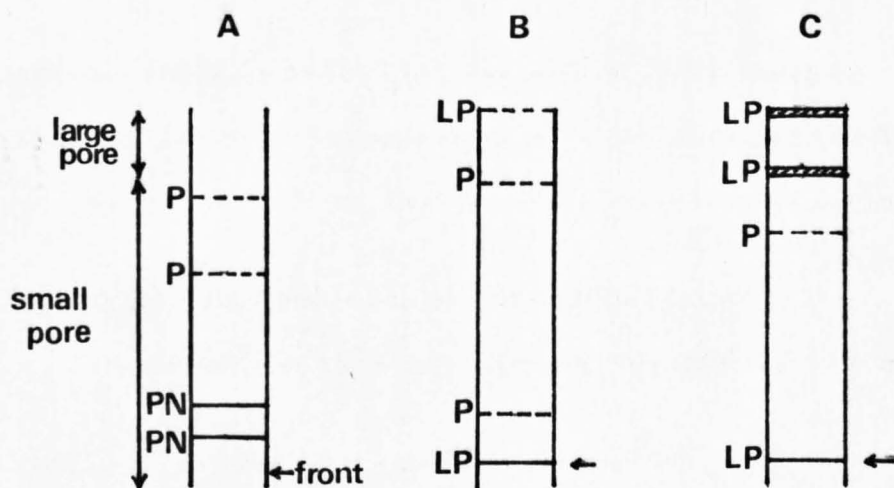
Details of the method used are given in paragraph 4.5.  
Gels were stained for protein, lipid or nucleotide.

- (A) light fraction from crude membrane
- (B) light fraction from purified membrane
- (C) heavy fraction from purified membrane

Key: P = stains for protein

L = " " lipid

N = " " nucleotide



slightly slower rate through the gradient than the heavy band produced by crude membrane.

4.14 The effect of  $Mg^{2+}$  on the release of protein from purified membrane during centrifugation on a sucrose gradient

The presence of  $Mg^{2+}$  caused a marked depression in the release of the nucleotide-protein complex from crude membrane during gel-filtration. Also  $Mg^{2+}$  promoted the ease with which crude membrane could be sedimented during ultracentrifugation, and was normally included in buffers during the final stages of the preparation prior to gel-filtration. Thus it was of interest to investigate the effect of  $Mg^{2+}$  on the low M.W. fraction released from purified membrane by sucrose gradient centrifugation.

Purified membrane of strain 1 was prepared from crude membrane by gel-filtration on Agarose A50m in the presence of Tris HCl buffer. No  $MgCl_2$  was included in this buffer. Aliquots of the purified membrane containing about 100 mg of protein were then dialysed for 14 hours at  $4^{\circ}C$  against  $0.05M$  Tris HCl pH 8.0 containing  $1mM$  or  $10mM$   $MgCl_2$ .

5-70% sucrose gradients containing Tris HCl buffer and  $1mM$  or  $10mM$   $MgCl_2$  were run as described in paragraph 4.4. Table 4.1 summarises the distribution of protein in the fractions obtained when the centrifuge tubes were emptied. The presence of  $Mg^{2+}$  does not appear to have affected the release of protein from purified

Table 4.1 The effect of  $Mg^{2+}$  on the release of protein from strain 1 purified membrane during sucrose gradient centrifugation.

Conc. of $Mg^{2+}$ mM	% Distribution of Protein	
	Heavy Band	Light Band
0	80	20
1	85	16
10	75	20



membrane. A further characterisation of this light membrane fraction (LMF) is described in paragraph 4.15.

#### 4.15 Characterisation of the protein released from purified membrane during sucrose gradient centrifugation

A volume of light membrane fraction (LMF) released from strain 1 purified membrane during gradient centrifugation in the absence of  $Mg^{2+}$  was dialysed against 0.05M Tris HCl pH 8.0 to remove sucrose, and concentrated using an ultrafilter fitted with a membrane which was only permeable to molecules of a molecular weight less than 1000.

Aliquots of the concentrate were investigated by gel-filtration on Sephadex and polyacrylamide disc-electrophoresis as described in Section I.

Electrophoresis revealed a prominent fast moving band which stained weakly for lipid and strongly for protein. An example of a gel is shown diagrammatically in Figure 4.6. The slow moving bands were not always detected and when present were stained only very faintly. A gel from the electrophoresis of the heavy fraction is also shown in Figure 4.6. This gel has the same distribution of bands as the purified membrane gel shown in Figure 4.3, including the fast moving band at the gel front.

Gel filtration on Sephadex G100 produced a single rather



broad peak in the column void volume indicating a M.W. greater than 150,000.

The amino acid analysis of LMF is shown in Table 4.2 with purified membrane and the nucleotide-protein complex released by gel-filtration of crude membrane on Agarose are also included for comparison. These results indicate that the fraction released on gradient centrifugation resembles the membrane in its overall apolar character rather than the nucleotide-protein complex which has a predominance of polar amino acids.

Amino Acids	Composition (mole %)		
	1.	2.	3.
Lysine	2.2	3.0	2.4
Histidine	1.2	1.6	1.8
Arginine	2.8	3.4	3.1
Aspartic Acid	9.1	16.5	12.4
Threonine	7.3	8.1	6.8
Serine	6.1	10.4	5.0
Glutamic Acid	8.9	11.1	10.4
Proline	4.7	1.8	3.8
Glycine	10.7	10.2	8.8
Alanine	10.7	10.0	10.1
$\frac{1}{2}$ Cystine	0.4	0.6	0.7
Valine	10.5	7.4	9.3
Methionine	1.6	0.8	1.7
Isoleucine	4.4	3.7	4.4
Leucine	9.8	6.0	8.4
Tyrosine	2.0	3.0	2.5
Phenylalanine	3.5	2.5	3.0

Table 4.2 Amino acid analyses of (1) purified strain 1 membrane, (2) nucleotide-protein complex released from strain 1 membrane by gel-filtration on Agarose A50 in the absence of  $Mg^{2+}$  and (3) LMF released from purified membrane by sucrose gradient centrifugation. (1) and (2) from Brown, Bellingham and Stevenson (1971). Results are expressed as moles AA per 100 moles.

#### 4.16 The effect of urea on purified membrane

In experiments carried out by RH Brown it has been shown that 8M urea caused the disaggregation of strain 1 membrane into fragments which could be separated by gel-filtration on Agarose A50m gel (Brown, Bellingham and Stevenson, 1971). Three fractions were obtained with the following recovery of protein (i) 65% residual membranous lipoprotein; (ii) 30% high M.W. protein; (iii) 5% low M.W. protein. Release of the latter two proteins could be suppressed to about 10% by the presence of 1mM  $Mg^{2+}$ .

In the experiments described in this section urea treated membranes were characterised by polyacrylamide gel electrophoresis. Samples of membrane were incubated with 8M urea for 3 hours at room temperature and then applied to 7% acrylamide gels containing 6M urea as described in paragraph 4.5. After staining the distribution of bands was identical to that obtained with purified membrane in the absence of urea except that the two mobile bands in the small pore gel were very much more prominent in the presence of urea. Preparations of strain 1M gave the same result as wild-type membrane. The distribution of the bands was not affected by the presence of 10mM  $Mg^{2+}$ .

Samples of the front band from strain 1 membrane incubated in the absence and presence of urea were isolated by quantitative P.G.E. (see paragraph 4.5) and examined by gel-filtration and

amino acid analysis. This fraction represented about 8% of the protein applied to the column after treatment with urea, and 3-5% in the absence of urea. Gel-filtration on Sephadex G25 indicated a M.W. of about 2500 for both fractions and amino acid analyses are shown in Table 4.3. For comparison the analyses of the fragments released by gel-filtration in the presence of urea are also included. The material isolated by PGE in the presence or absence of urea and the low M.W. fraction from gel-filtration bear a striking similarity to each other particularly with reference to the relative proportions of hydrophilic and apolar amino acids. These fractions appear to be considerably more polar in character than the purified membrane. Also the high M.W. material released in the presence of urea, although more polar than the membrane is rather less polar than the low M.W. fractions.

Amino Acids	Composition (mole %)				
	1.	2.	3.	4.	5.
Lysine	2.2	6.3	6.1	2.0	6.2
Histidine	1.2	2.5	2.5	1.2	2.3
Arginine	2.8	2.0	2.2	2.5	2.0
Aspartic acid	9.1	10.0	9.9	15.9	10.1
Threonine	7.3	6.8	6.8	9.6	6.7
Serine	6.1	10.6	10.5	8.0	10.4
Glutamic acid	8.9	16.1	15.8	14.6	16.6
Proline	4.7	3.4	3.1	3.6	3.6
Glycine	10.7	15.6	15.8	10.3	15.8
Alanine	10.7	10.0	9.6	9.9	9.4
$\frac{1}{2}$ Cystine	0.4	0	0	0	0
Valine	10.5	4.5	4.4	5.2	4.4
Methionine	1.6	1.4	1.4	0.9	1.3
Ileucine	4.4	3.5	3.7	4.2	3.6
Leucine	9.8	4.1	4.6	6.0	4.3
Tyrosine	2.0	1.5	1.9	3.6	1.6
Phenylalamine	3.5	1.9	1.7	2.0	2.0

Table 4.3 Amino acid analyses of (1) purified strain 1 membrane. (2) and (3) front band obtained from preparative PGE of membrane in the absence and presence of urea respectively. (4) and (5) respectively high M.W. and low M.W. fractions released by urea from membrane and separated by gel-filtration on Agarose A50m. Results are expressed as mole % i.e. moles per 100 moles. (1), (4) and (5) from Brown, Bellingham and Stevenson (1971).

### Section III : Discussion

Electronmicrographs of the crude membrane preparations from strain 1 and 1M show substantial amounts of material with the typical appearances of triple layered unit membrane structures. The purified membrane samples obtained by gel-filtration on Agarose also show considerable amounts of membrane fragments although the prominent vesicles which were evident in crude membrane em's appear to have been disrupted and the fragments tend to be smaller than in the crude membrane preparations. This is particularly so in the case of strain 1M preparations. The disruption into smaller membranous fragments may account for the "double peak" seen on density gradients of purified membrane. If this is so, then the light membrane fraction (LMF) would resemble purified membrane in its general composition. The results characterising LMF tend to support this hypothesis. Table 4.4 summarises the characteristics of LMF and the other preparations isolated in experiments described in chapter IV.

The relative degree of polar or non-polar character of a protein depends on the proportion of both the hydrophilic (H) and hydrophobic or apolar (A) amino acids (see Table 4.5).

Fraction	Recovery (%)	Effect of Mg <sup>2+</sup> (+ or -)	M.W.	H/A
I Crude membrane	*	+	*	1.6
II Purified membrane	65	+	50 x 10 <sup>6</sup>	1.3
III Nucleotide-protein complex	35	+	2,000	2.7
IV LMF	15-20	-	150,000	1.6
V Fractions released i) 65 from II by urea ii) 30 on Agarose <sup>a</sup> iii) 5		* + +	50 x 10 <sup>6</sup> 800,000 2,300	1.2 2.9 3.5
VI Fractions isolated by PGE of II				
+ urea iv) 8		-	2,500	3.4
- urea v) 3-5		-	2,500	3.5

Table 4.4 Summary of the characteristics of fractions isolated from crude and purified membranes of H.salinarium strain 1. See text of Chapter IV for details of preparations.

\* No data

<sup>a</sup> Data from Brown, Bellingham and Stevenson (1971).

(H/A) = ratio of hydrophilic amino acids to apolar amino acids (mole %). See Table 4.5.

Hydrophilic	Apolar
Asp	Val
Glu	Ile
Ser	Leu
Thr	Phe
Arg	Met
Lys	
His	

Table 4.5 Sets of amino acids used to calculate the ratio of hydrophilic to apolar amino acids (H/A). (Hatch and Bruce, 1968).

Thus the ratio H/A may be used to express the overall polar character of a protein and may be related to the environment to which a particular moiety is exposed. (Hatch and Bruce, 1968).

LMF has a similar H/A value to the crude and purified membrane preparations implying a strong similarity in degree of apolar character. In contrast the nucleotide-protein complex released from crude membrane on Agarose and sucrose gradients has a much higher H/A value. There does not seem to be any similarity between LMF and this fraction since LMF also has a very much higher M.W., different PGE profile, lower  $\frac{A_{254}}{A_{280}}$  ratio, and its release is not affected by  $Mg^{2+}$ . This latter finding is perhaps rather surprising since both crude and purified membrane aggregate in



the presence of  $Mg^{2+}$  into sheets or vesicles (Brown, 1969). Thus it is possible that LMF represents a fraction which is essentially similar to the remaining lipoprotein membrane, but lacks the moieties which are involved in the interactions with  $Mg^{2+}$ . The presence of cytochrome and carotenoid was also not detected in LMF from strain 1 membrane.

On the other hand the release of the nucleotide-protein complex can be reduced by the presence of  $Mg^{2+}$ . The separation of this material observed on gel-filtration of crude membrane in the absence of  $Mg^{2+}$  may be due to the opening of vesicles and release of trapped cytoplasmic material. However, it is possible that the nucleotide-protein may be of genuine membrane origin. Labile membrane-ribosomal complexes have been reported (Schlessinger, 1964; Razin, 1967; Coleman, 1968) and in H. salinarium any residual cytoplasmic ribosomes would be expected to disaggregate (Bayley, 1966) and therefore not sediment under the salt-free conditions used in the preparation of crude membrane.

However, polar, nucleotide-free,  $Mg^{2+}$  sensitive protein fractions have been isolated from purified strain 1 membrane by treatment with urea. Low and high M.W. fractions representing about one third of the membrane protein were isolated by gel-filtration in the presence of urea (Brown, Bellingham and Stevenson, 1971) (see Table 4.4). The release of these fractions was markedly reduced by the presence of  $Mg^{2+}$ . However,  $Mg^{2+}$  did

not affect the release of a similar low M.W. fraction which could be isolated in the absence of urea by preparative PGE. When 6M urea was incorporated into the gel release of this material was enhanced and this effect was not decreased by the presence of  $Mg^{2+}$ . Amino acid analysis indicated that this protein was polar and gave an identical H/A ratio to the low M.W. fraction isolated by gel-filtration with urea containing buffer.

The high degree of polar character in this fraction combined with a low M.W. probably facilitates separation from the membrane by PGE. Thus the binding effect of  $Mg^{2+}$  would tend to be reversed. This is indicated by the fact that release of this protein on PGE is increased only marginally by the presence of urea.

The residue of the urea treated membrane isolated by gel-filtration was of very high M.W. and distinctly apolar, more so than purified or crude membrane. This material is presumably aggregated lipoprotein, possibly in a membrane like configuration. Unfortunately the effect of  $Mg^{2+}$  on this material was not investigated, thus it is not known if it behaves in a manner similar to the crude or purified membrane preparations. It is possible that  $Mg^{2+}$  would have no effect since the polar material, the binding of which is influenced by the presence of  $Mg^{2+}$ , would have been removed by the urea treatment.

Thus it appears that the bulk of the membrane protein is

essentially apolar and combined with a lower proportion of more polar material. The binding of this material may be disaggregated with urea or promoted with  $Mg^{2+}$ . In the following chapter a detailed investigation of the membrane lipid and protein is described.

## Chapter V : The chemical composition of purified membrane

### Introduction

The isolation of pure cell membranes from the common Gram-negative bacteria is impeded by the presence of a complex cell wall (Salton, 1967). Spheroplasts prepared by lysozyme digestion remain surrounded by lipopolysaccharide and lipoprotein layers. These layers are morphologically similar to the cell membrane and difficult to separate from it, although attempts to achieve this by differential centrifugation have been made (Miura and Mizushima, 1968). An alternative approach has been to remove the lipopolysaccharide layer first with E.D.T.A. followed by detergent to lyse the resulting spheroplast (Birdsell and Cota-Robles, 1968). However, it is possible that the detergent might solubilise the membrane to some extent resulting in a loss of lipoprotein components. The instability of the cell wall of Halobacteria in low salt concentrations offers a unique opportunity for the isolation of cell membranes free from contaminating cell wall components.

The composition of membranes from many different sources have been investigated e.g. myelin, erythrocytes, mitochondria, liver cells, chloroplasts and bacteria, (Korn, 1969a). Ratios of protein and lipid tend to differ widely, sterol is not always present and the nature of the polar lipids is highly variable, especially with respect to fatty acid composition. Myelin contains high concentrations

of saturated and  $\alpha$ -hydroxy fatty acids, membranes from chloroplasts uniquely contain 3-hexadecenoate, fatty acids of Gram-positive bacteria are almost exclusively branched chain (Korn, 1969a) whilst in Halobacteria fatty acids are totally replaced by phytol alcohol (Kates et al 1965). This feature distinguishes them markedly from other bacteria.

The structural and functional significance of these variations and the interactions of membrane protein and lipid are not clearly understood. Thus the membranes of organisms like Halobacteria occurring naturally in extreme environments are of potential interest as experimental tools for the correlation of molecular structure and function. With this in view the chemical composition of wild-type and mutant membranes prepared by the methods described in Chapter IV were investigated as a preliminary to subfractionation.

## Section I : Methods

### 5.1 Extraction of lipids

Total lipids were extracted from preparations of purified membrane by the chloroform : methanol method of Bligh and Dyer (1959). In place of the filtration step described in the original procedure the extracted protein was separated by centrifuging the homogenate in a Sorvall RC2-B centrifuge. A GSA rotor was used with glass bottles about half-full and spun at  $2,000 \times g$  for 10-15 minutes at  $20^{\circ}\text{C}$ . Coagulated denatured protein collected at the interface of the aqueous and chloroform layers. The chloroform layer was removed carefully with a pipette and the upper layer re-extracted at least twice. The chloroform from each extraction was combined, filtered through glass wool, and dried under a stream of nitrogen. The residue was re-extracted with dry chloroform until of constant weight. Exposure of lipid samples to strong light was avoided and the final residue was stored under vacuum at  $-20^{\circ}\text{C}$  in a dessiccator containing silica gel.

### 5.2 Fractionation of total lipid extracts

Total lipid extracts were fractionated by the use of column and thin layer chromatography. The procedures used were based on those described by Brown and Stevenson (1970).

D.E.A.E. cellulose chromatography: This technique was used

to separate the lipids into polar and non-polar fractions. The use of D.E.A.E. cellulose for the fractionation of lipids has previously been described by Rouser et al (1965). D.E.A.E. cellulose (Whatman DE-50) was pre-cycled with 3 volumes of  $1M$  HCl and  $0.1M$  KOH, then washed with 3 volumes each of water, acetic acid and methanol and finally dried in a dessiccator containing solid KOH. About 5 g of dried resin was converted to the acetate form by stirring into acetic acid and leaving overnight at room temperature. A column of 1 cm internal diameter was then set up using all of the resin and washed first with 4 volumes of methanol and then 4 volumes of chloroform. A solution containing about 100 mg of lipid dissolved in 1-2 ml of chloroform was applied to the top of the column. Non-polar lipids were eluted with chloroform, polar lipids with methanol-chloroform (1:2, v/v) and chloroform-methanol-28% (w/v) aqueous ammonia (8:4:1, by volume). By this procedure at least 96% of the lipid applied to the column was recovered.

Decalso chromatography: About 5 g of "Decalso-F" (Permutit) ion exchange resin was suspended in a small volume of light petroleum ( $40^{\circ}$ - $60^{\circ}$ ) and packed in a column of 1.5 cm diameter. The column was washed with several volumes of light petroleum prior to applying 10 mg of non-polar lipids from the D.E.A.E. cellulose fractionation dissolved in light petroleum. Neutral lipids were eluted with 75 ml of light petroleum and menaquinone was recovered with 50 ml of 4% (v/v) diethyl ether in light

petroleum. Most of the remaining lipid was eluted with 40 ml of methylal (dimethoxymethane).

Thin layer chromatography: T.L.C. of lipid extracts was carried out routinely using 20 cm x 20 cm plates of silica gel (MN-Silica Gel N, Macherey, Nagel and Co) 0.25 mm thick. The resolution of the plates was improved by scoring the dry gel along the length of the plate at right angles to the direction of the solvent flow, (Blank, Schmidt and Privett, 1964). The plates were activated by heating at 110°C for 1 hour and run in glass tanks lined with filter paper. The tanks were allowed to equilibrate with solvent for at least 36 hours. Lipid samples applied to the plates were dissolved in chloroform.

For the analysis of total lipids or phospholipids the plates were developed using a chloroform-methanol-water system (65:25:4, by volume). Neutral lipids were separated using chloroform: methanol (4:1, by volume) or diethyl-ether:light petroleum (40-60°): acetic acid (30:170:1, by volume). Spots were visualised by charring with a spray of 50% v/v  $H_2SO_4$  or iodine vapour. Cis-glycols were detected by the periodate-Schiff reaction, (Sastry and Kates, 1964).

5.3 Estimation of menaquinone and carotenoid: Carotenoid was determined as described in paragraph 4.8. Menaquinone in fractions from the Decalso column was estimated from the absorption at 249 nm in cyclohexane solution using an  $E_{1\text{ cm}}^{1\%}$  value of 264 (Bishop, Pandya



and King, 1962).

5.4 Gel-filtration on Sephadex LH-20: The dissolution of membranes in suitable organic solvents followed by the subsequent isolation of lipid free proteins by gel-filtration on the organophilic polydextran LH-20 (Pharmacia) has previously been described by Zahler and Wallach (1967).

Purified Halobacterium salinarum membrane was fully soluble when treated with 90% 2-chloroethanol in 0.01M HCl. Samples containing about 40 mg of protein were applied to the top of a 2.5 cm x 30 cm LH-20 column previously equilibrated with 90% 2-chloroethanol in 0.01M HCl. The protein was eluted using the same solvent at a flow rate of 15 ml per hour. The effluent was collected in 2.0 ml fractions using a central fraction collector and the absorbancy monitored at 254 nm with an LKB Uvicord and recorder.

5.5 Preparation of structural protein: Structural protein (SP) was prepared from purified membranes of strain 1 and 1M by two methods:

SP extracted with mixed detergents and ammonium sulphate: The procedure described by Criddle et al (1962) was followed with the modifications and additional purification stages used by Lenaz et al (1968) for the extraction of SP from mitochondria. 20 mg of membrane in 2.0 ml of water were treated with 0.4 ml of a mixed detergent solution (MAD) containing SDS (37.5 mg/ml), sodium

deoxycholate (DOC, 100 mg/ml) and sodium cholate (50 mg/ml). Residual material was removed by centrifuging at 20,000 x g for 10 minutes. After the pH had been adjusted to pH 9.0 with KOH a few small crystals of  $\text{Na}_2\text{S}_2\text{O}_4$  were added to reduce cytochromes. The solution was then made 12% of saturation with respect to ammonium sulphate, adjusted to pH 9.0 and left at 4°C for 15 hours before centrifuging. The pellet obtained was washed with 0.25M sucrose. Lipids and detergents were removed by the method of Criddle et al (1962) using n-butanol in the presence of ammonium sulphate and DOC followed by many washings with methanol. The resulting crude SP was purified by the extraction of residual haem proteins and urea soluble material by the methods of Lenaz et al (1968). The final urea insoluble residue was washed with water and lyophilised.

SP extracted with HCl: This method was based on the acid extraction of SP from Yeast mitochondria described by Lejsek and Lusena (1969). A pellet of about 40 mg of purified membrane was treated with 5 ml of 0.1M HCl and stirred at 4°C for 14 hours. The resulting suspension was centrifuged at 150,000 x g for 1 hour, resuspended in 0.1M HCl and respun. The supernatants were combined and dialysed for 14 hours at 4°C, firstly against distilled water and then 0.05M Tris HCl pH 7.5. The resulting precipitate was sedimented, washed with water and lyophilised.

## 5.6 Polyacrylamide disc electrophoresis:

Lipid-free membrane protein: Gels containing 5M urea and 0.1% SDS were set up as previously described in paragraph 4.5. The pH 8.9 borate electrode buffer also contained 0.1% SDS. Protein samples dissolved in 6M urea, 0.1% SDS and 0.1% 2-mercapto-ethanol were applied to the gels and electrophoresed for 10 minutes at 3mA per tube and 2 hours at 5mA per tube.

### SP extracted with mixed detergent and ammonium sulphate:

Prior to electrophoresis lyophilised SP was dissolved in a solution containing 4 g phenol, 2.4 g of urea, 2 ml glacial acetic acid and 2 ml water (Lenaz et al, 1968). 7% acrylamide gels containing urea and acetic acid were prepared according to the method of Takayama et al (1964) as modified by Baum et al (1967). Electrophoresis was carried out for 2½ hours at 5 mA per tube.

SP extracted with 0.1N HCl: Electrophoresis was carried out by the procedure of Lejsek and Lusena (1969). The urea containing gels were composed of a 3.5% acrylamide stacking gel and a 7% acrylamide running gel of pH 5.3 and 3.3 respectively. The electrode buffer was 74 mM glycine adjusted to pH 3.75 with formic acid. Samples of SP dissolved in 8M urea pH 3.3 were applied to the gels and electrophoresis run for 15 minutes at 3 mA per tube and 2½ hours at 5 mA per tube.

In each of the three methods described the gels were fixed and stained for protein according to the method of Chrambach et al (1967).

#### 5.7 Cultivation of cells in $^{32}\text{P}$ and $^{14}\text{C}$ labelled media

Radioisotope labelled compounds were obtained from the Radiochemical Centre, Amersham. Early logarithmic phase cultures grown on the media and under the conditions described in paragraph 2.2 were inoculated with labelled substrates.

2.0 mCi of  $^{32}\text{P}$  sodium orthophosphate in phosphate buffer were added per litre of culture 8 hours after growth was started with a 10% inoculum of actively growing cells. The cultures were harvested after 60 hours growth.

In other experiments 50  $\mu\text{Ci}$  of glycerol  $-^{14}\text{C}$  (U), sodium acetate  $-^{14}\text{C}$  (U) or sodium mevalonate  $-2: ^{14}\text{C}$  were added per 100 ml of medium. Mevalonate was obtained as a solution of mevalonic acid lactone in benzene. This was evaporated with a freeze-dryer and the residue dissolved in 0.01M sodium phosphate pH 7.0.

5.8 Scintillation counting of  $^{14}\text{C}$  and  $^{32}\text{P}$ : The level of radioactivity in material labelled with  $^{14}\text{C}$  or  $^{32}\text{P}$  was measured using a Packard Tri-Carb Liquid Scintillation Spectrophotometer.

Samples of lipid separated by T.L.C. were counted in the presence of silica gel by the procedure of Snyder, (1964). Spots

on the T.L.C. plates were visualised by charring with iodine vapour and then scraped into counting vials. A thixotropic gelling agent was then added, "Cab-O-Sil", 4% (w/w), (Cabot Corp.) and 10 ml of a dioxane-water based scintillation fluid. The suspension was shaken to disperse particles of adsorbent before the medium gelled. The scintillation fluid contained 7 g PPO (2,5-diphenyloxazole), 0.3 g dimethyl POPOP (1,4-bis-2-(4-methyl-5-phenyloxazolyl)-Benzene, 100 g naphthalene, dissolved and diluted to 1 litre with dioxane and then mixed with water in the ratio 15:3. This solvent system used in conjunction with iodine vapour minimises quenching due to charring and the adsorption of polar compounds by the silica gel particles.

Aqueous solutions of lipoprotein obtained by gel-filtration of disaggregated membrane were dissolved in a toluene-Triton based scintillation fluid previously described by Paterson and Greene (1965). PPO, 8 g and dimethyl POPOP, 0.1 g were dissolved in 2 litres toluene and then mixed with Triton X-100 (B.D.H.) in the ratio 2:1. Some of the fractions obtained by gel-filtration contained carotenoid. The severe quenching effect caused by this compound was reduced by the addition of a few small crystals of sodium borohydride to all fractions to be counted (Turner, 1967). 0.1 ml aliquots of the decolourised solutions were added to 10 ml of scintillation fluid and the vials shaken before reading.

## Section II : Results

### 5.9 Gross composition of the cell membranes of *H. salinarium* strains 1 and 1M

Crude and purified membrane samples were prepared from *H. salinarium* strains 1 and 1M as described in Chapter IV. It has been shown that the cell lipid, carotenoid, menaquinone and cytochromes are localised in the cell membrane (Brown, Bellingham and Stevenson, 1971). The levels of lipid, carotenoid, menaquinone and protein were determined by the procedures described in 'Methods'. Cytochromes were estimated from difference spectra by measuring  $A_{427\text{nm}} - A_{480\text{nm}}$  after reduction by sodium dithionite and expressed as arbitrary units (a.u.) per g dry weight of membrane (Bednar, 1965). The results obtained are summarised in Table 5.1.

The ratio of total protein to total lipid in both strains is the same. However, the red carotenoid, a bacterioruberin (Liaaen Jensen, 1960) is absent from the colourless mutant and the level of menaquinone is also about 50% lower in the mutant. The amount of cytochromes reduced by dithionite are about the same in both strains.

### 5.10 Extraction and isolation of the membrane lipids

Total lipid extracts from strain 1 and 1M were separated by

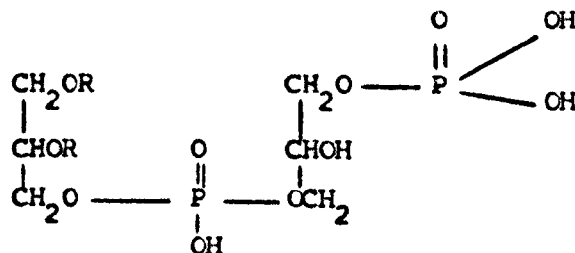
	Composition of purified membrane	
	Strain 1	Strain 1M
Lipid % dry weight	51	50
Protein % dry weight	49	50
Carotenoid % dry weight	0.4	0
Menaquinone % dry weight	1.0	0.43
Cytochromes a.u./g membrane protein	0.14	0.16

Table 5.1 The levels of the main constituents of the cell membranes of H. salinarium strain 1 and 1M.

D.E.A.E. cellulose chromatography. Non-polar lipids were washed from the column with  $\text{CHCl}_3$  and constituted about 6% of the total lipid. The remaining polar lipids were eluted with a mixture containing  $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{NH}_3$ . In both strains 90% - 93% of the lipid was polar. This result agrees with that obtained by Brown and Stevenson (1971) when they examined the total lipid from strain 1.

The lipid fractions were also separated by T.L.C. When the plates were developed with a  $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}$  mixture the non-polar lipids migrated at the solvent front and the polar lipids were separated into 5 or 6 constituents. The result of such a chromatoplate using total lipid extract is shown in Figure 5.1. A lipid extract of Halobacterium cutirubrum was also included for comparison since identification of some of the polar lipids present has been reported, Sehgal et al (1962) and Kates et al (1963), (1965). These workers reported that nearly all of the lipid in H. cutirubrum was phospholipid and that a single di-ether analogue of diphosphatidyl glycerophosphate (Structure I) constituted about 73% of the total lipid.

Structure I



R = 3,7,11,15-tetramethyl hexadecyl (dihydrophytyl)



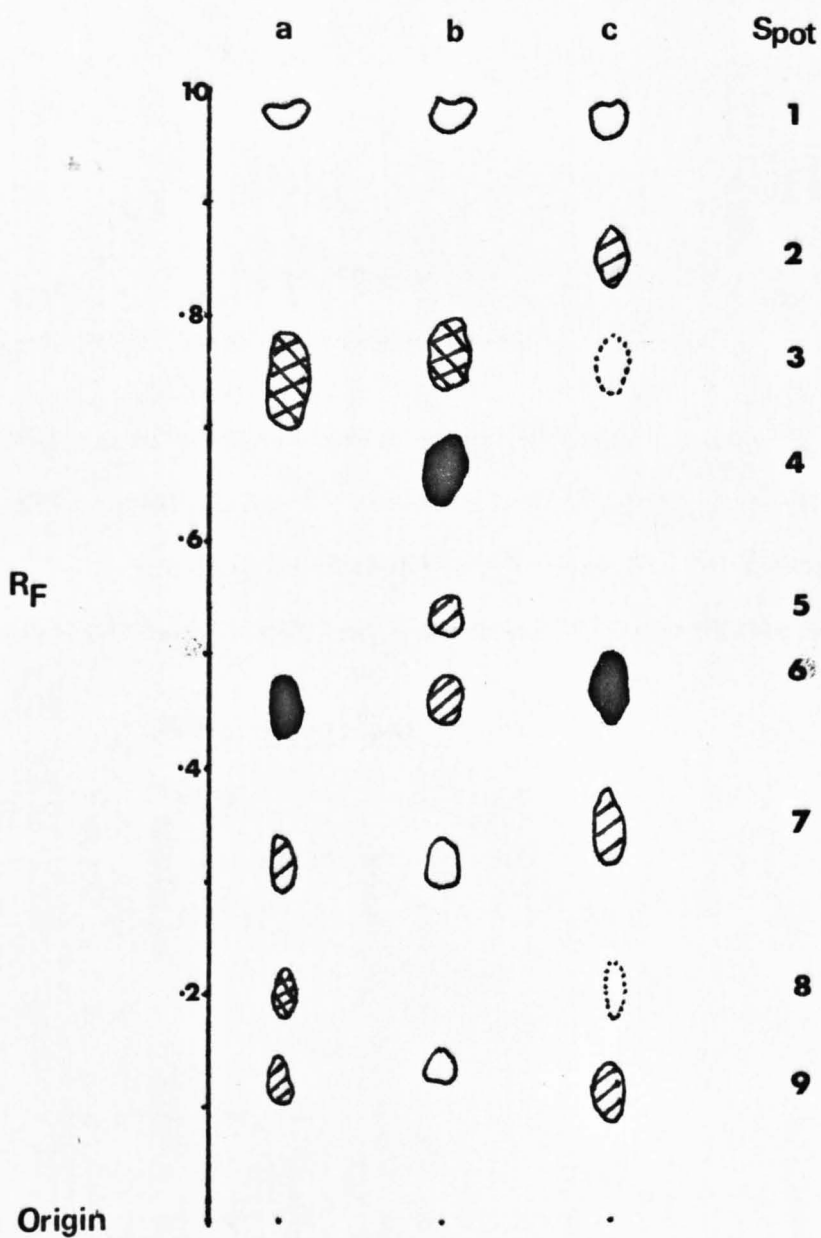
Figure 5.1

Thin-layer chromatography of total lipid extracts

Plates were developed in a solvent containing  $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}$  (65:25:4). Spots were visualised by charring with 40%  $\text{H}_2\text{SO}_4$ . The intensity of the spots is indicated by shading:

Block > crossed line > diagonal line > open line > dotted line

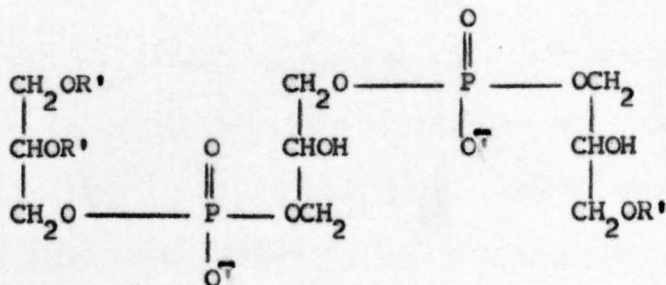
- (a) H. salinarium strain 1
- (b) " strain 1M
- (c) H. cutirubrum



They also suggested that a malonyl-CoA system for the synthesis of unbranched fatty acids was absent and apparently replaced by a mevalonate system for the synthesis of isoprenoid chains.

All of the polar lipids detected in H. salinarium strains 1 and 1M incorporated  $^{32}\text{P}$  as shown diagrammatically in Figure 5.2. The relative proportions of the phospho-lipids as indicated by visual examination after charring the surface of the plate with a spray of 40%  $\text{H}_2\text{SO}_4$  are indicated in Figure 5.1. Spot 6 was the most intense in both strain 1 and H. cutirubrum but somewhat less prominent in strain 1M. This spot probably corresponds to the major constituent of H. cutirubrum phospholipid (Structure I) described by Kates et al (1965). Spot 3 was prominent in strain 1 and 1M, but not confirmed in H. cutirubrum. Brown and Stevenson (1971) suggested that this phospholipid might be an ether analogue of 1,3-diglycerophosphoryl glycerol (Structure II).

Structure II



Structure I and possibly Structure II contain isoprenoid chains. When H. salinarium was grown on a medium containing  $\text{[2-}^{14}\text{C]}$  mevalonate, label was readily incorporated into the membrane lipids of both strains. Incorporation was significantly higher than when the growth medium was supplemented with  $^{14}\text{C}$ -(u) acetate or glycerol (see Table 5.2.) Strain 1M incorporated considerably more label than strain 1 when grown on mevalonate or glycerol.

Nearly all of the  $^{14}\text{C}$  was incorporated into spots 3 and 6 in the case of mevalonate, but distributed more or less unspecifically when labelled acetate and glycerol were used (see Figure 5.2.) Bearing in mind the relevance of mevalonate in isoprenoid synthesis this suggests that spots 3 and 6 contain isoprenoid chains, thus supporting the suggestion that spot 6 in both H. salinarium strains corresponds to structure I. Likewise spot 3 may also contain isoprenoid chains as the alkyl groups of Structure II.

Spots 4 and 5 were only present in strain 1M lipid. Spot 4 was the most prominent of the mutant phospholipids and significantly did not incorporate  $\text{[2-}^{14}\text{C]}$  mevalonate. Also it gave no reaction with a periodate-Schiff spray for cis-glycols. Spot 1 in strain 1 and 1M corresponded with non-polar lipid fractions obtained from D.E.A.E. chromatography.

Source of $^{14}\text{C}$	Percentage incorporation	
	Strain 1	Strain 1M
Mevalonate	30.0	48.9
Acetate	3.0	3.0
Glycerol	2.7	4.1

Table 5.2 Incorporation of radioisotope label into membrane lipids.

Figure 5.2

A diagrammatic representation of the distribution of radio-  
active label detected in components obtained by T.L.C. of total  
lipid extracts of H. salinarium strains 1 and 1M.

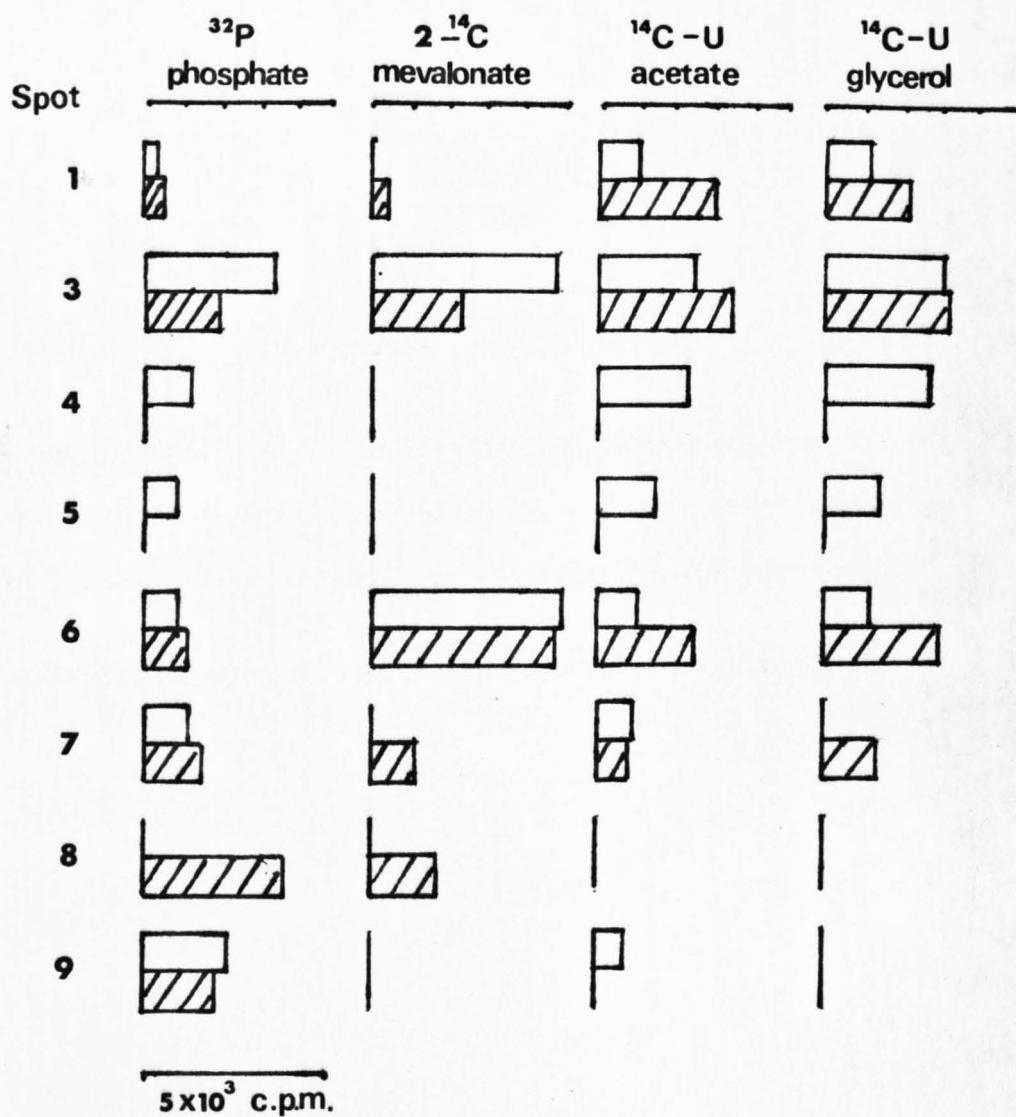
Experimental details are given in paragraphs 5.8 and 5.9.

Strain 1



Strain 1M





The non-polar lipids of strain 1 and 1M which were eluted from the D.E.A.E. cellulose column with chloroform were further separated by chromatography on a Decalso column. Approximately 40%-50% of the neutral lipid applied was removed by light petroleum (40°-60°C). On T.L.C. using ether:light petroleum:acetic acid solvent a predominant, fast moving component was obtained with both strains (see Figure 5.3). An i.r. spectrum of the neutral lipid from strain 1 indicated that it was mainly hydrocarbon (Brown, 1969). The remaining spots gave a similar distribution on the T.L.C. plate but were present to varying extents in different extractions from both strains. U.V. spectra of the light petroleum fractions are shown in Figure 5.4. These indicate that at least one component of strain 1 lipid which peaks at 318 nm and 330 nm is absent from the mutant.

The lipid remaining on the Decalso column was removed by 2% or 4% diethyl ether in light petroleum or methylal. The ether fraction contained menaquinone. In strain 1 98% of the menaquinone was removed by the 4% ether fraction, but in the mutant, menaquinone was only detected in the 2% ether fraction. Figure 5.5 shows u.v. spectra of these fractions. Menaquinone of strain 1 has previously been purified and shown by paper chromatography to be identical to menaquinone-8 (Stevenson, 1965).



### Figure 5.3

#### Thin-layer chromatography of neutral lipids fractionated on a Decalso column

The light petroleum fraction was separated using a solvent containing ether:light petroleum:acetic acid (30:170:1) and the methylal fraction with a solvent of  $\text{CHCl}_3$ : $\text{CH}_3\text{OH}$  (4:1). Details are given in paragraph 5.2. The intensity of the spots is indicated by shading:

Diagonal line > open line > dotted line

- (a) Strain 1 light petroleum fraction
- (b) Strain 1M " " "
- (c) Strain 1 methylal fraction
- (d) Strain 1M " "

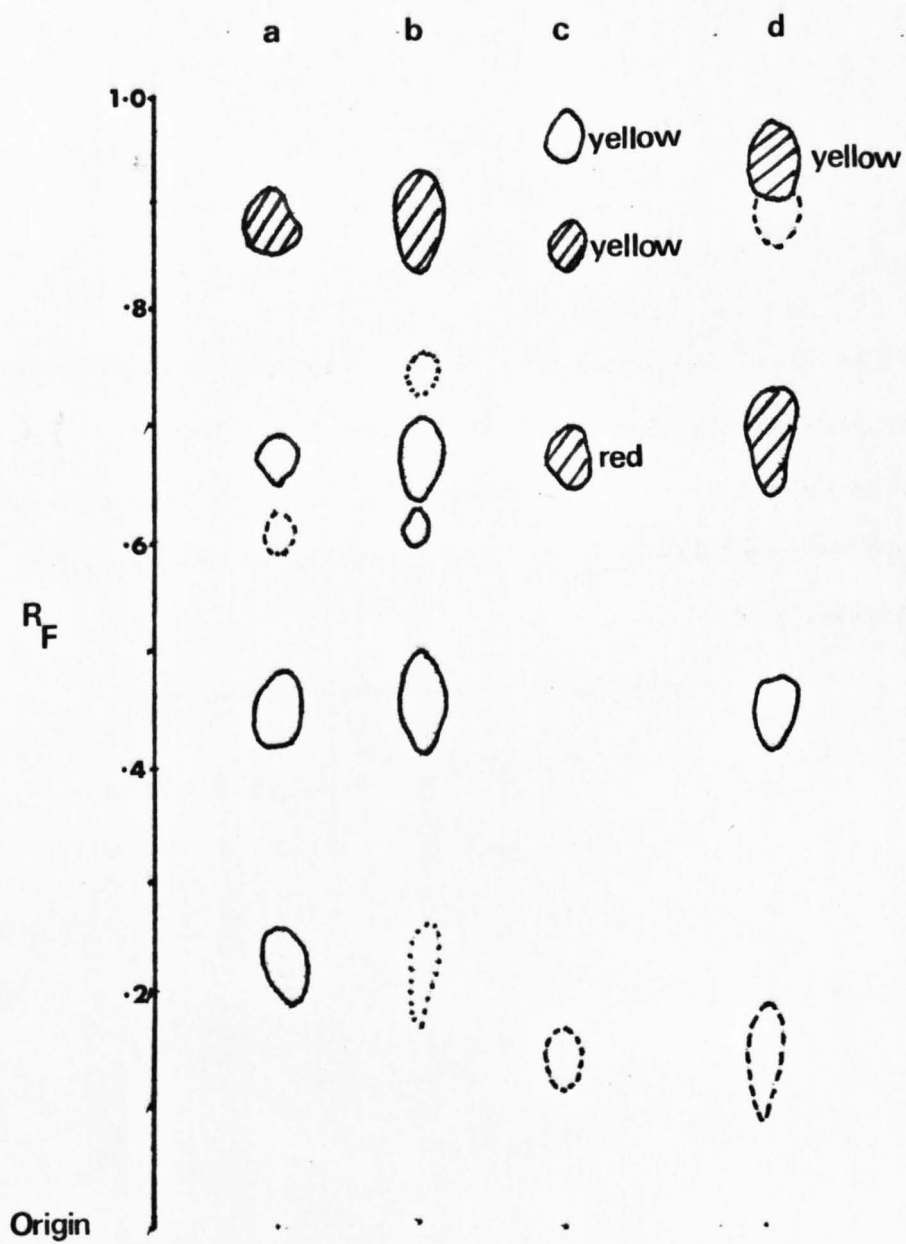


Figure 5.4

U.V. spectra of strain 1 and 1M neutral lipid obtained in the light petroleum eluate from a Decalso column.

The lipids were dissolved in cyclohexane solution.

strain 1    2.0 mg/ml

strain 1M   1.2 mg/ml

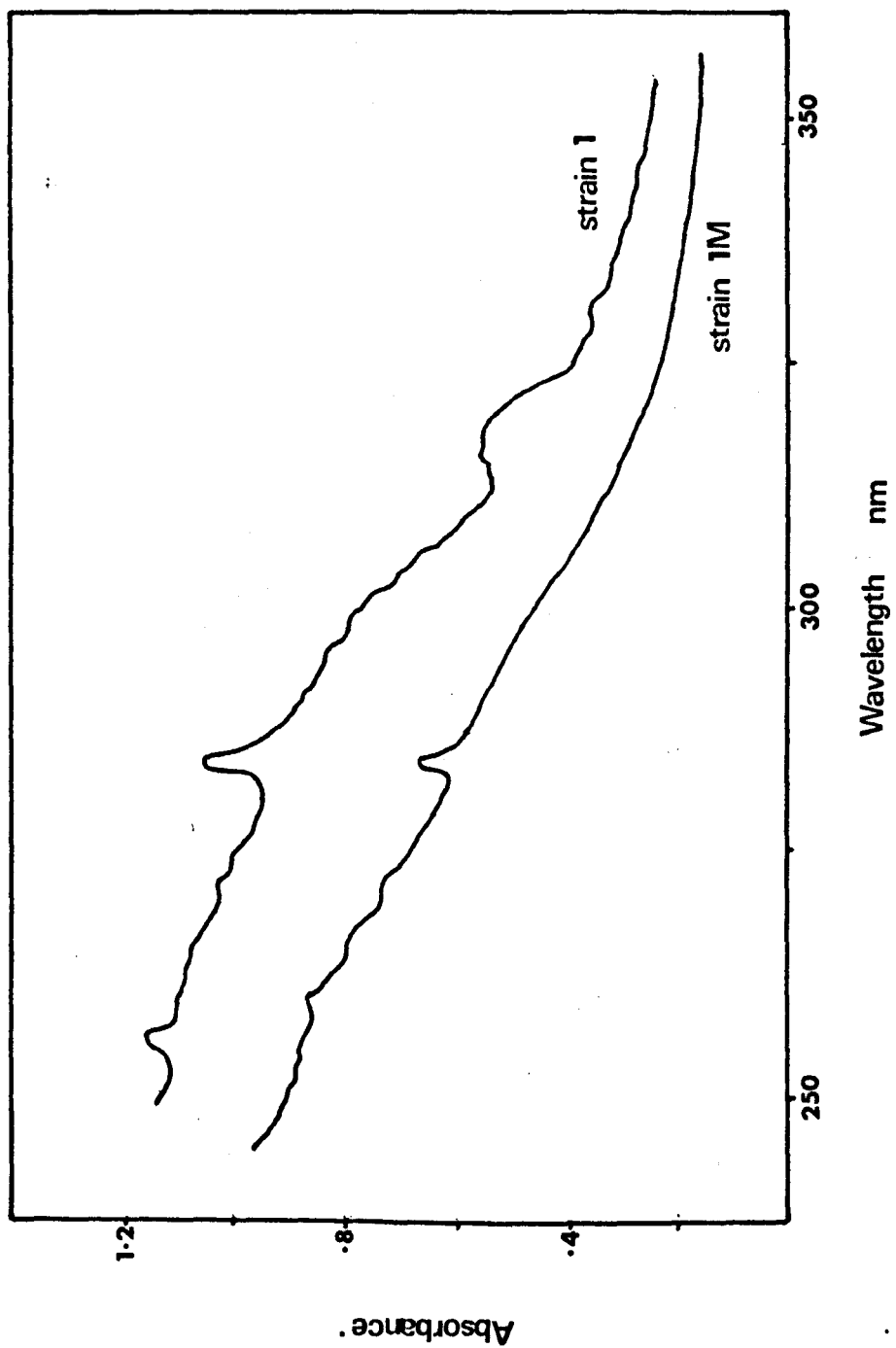


Figure 5.5

U.V. spectra of menaquinone containing fraction obtained by chromatography on Decalso of strain 1 and 1M lipid extracts.

(a) Spectra were measured in cyclohexane.

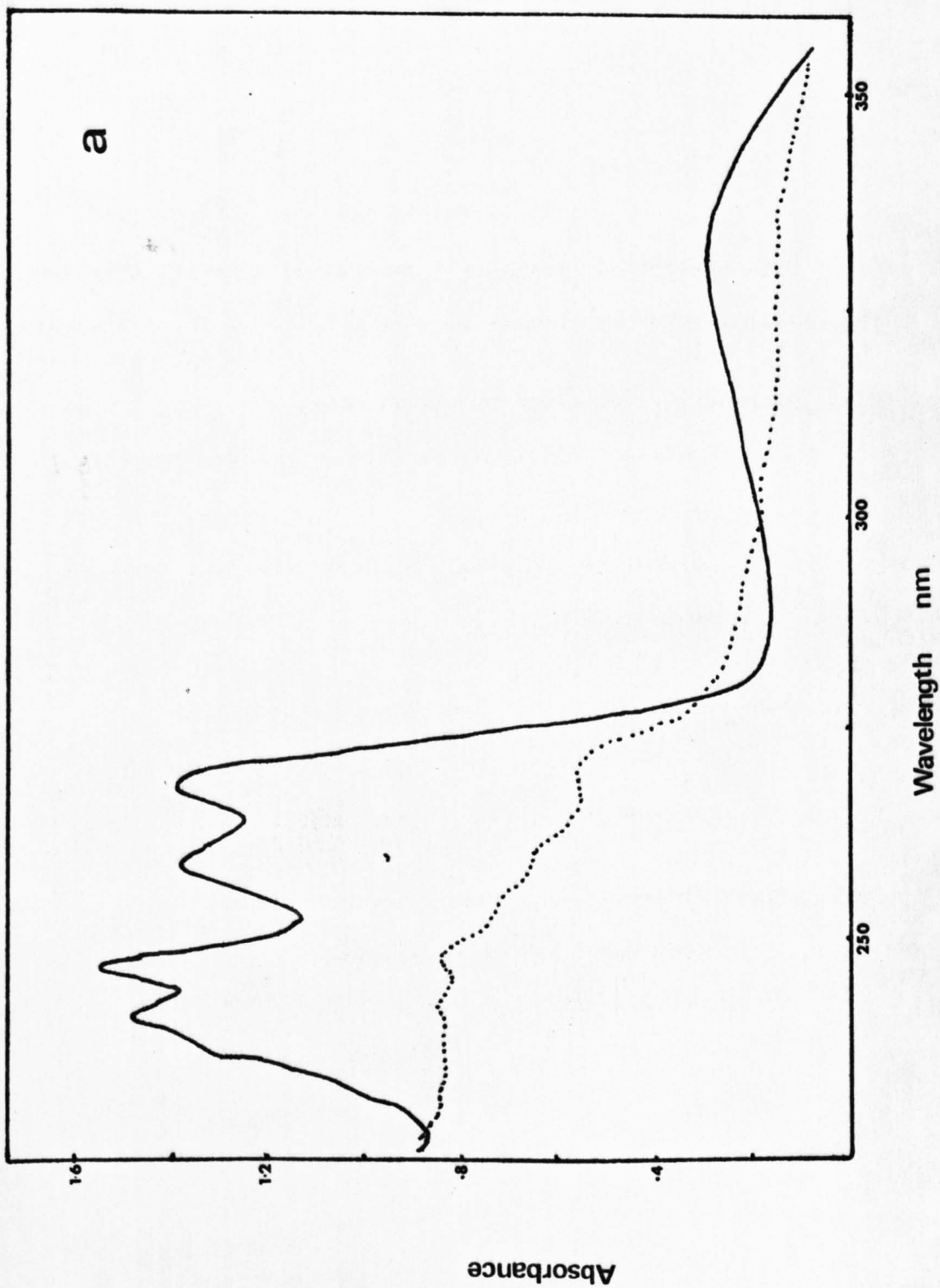
- strain 1 (4% ether in light petroleum fraction, 0.2 mg/ml).
- strain 1M (2% ether in light petroleum fraction, 0.63 mg/ml).

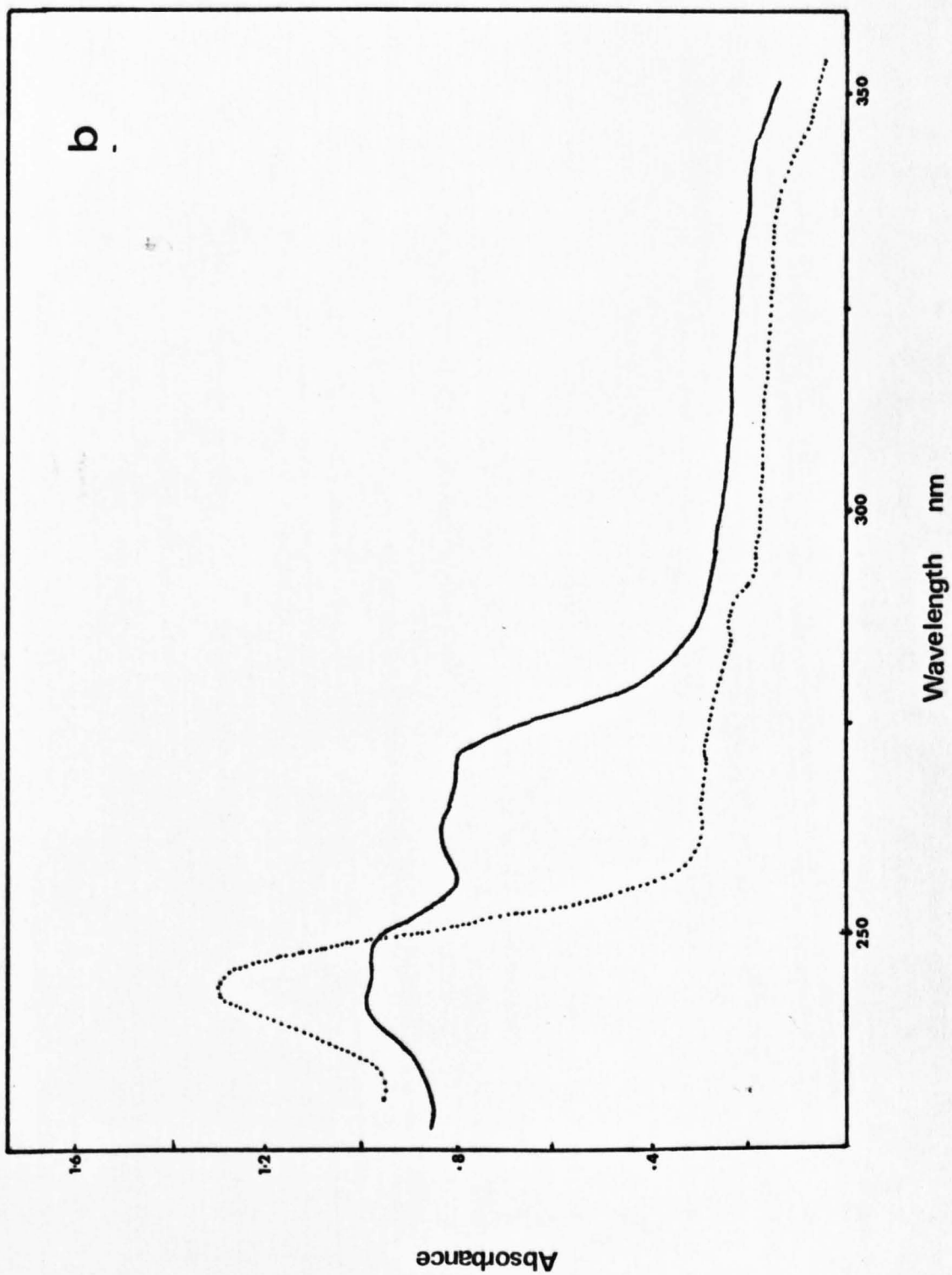
(b) Strain 1 menaquinone containing fraction.

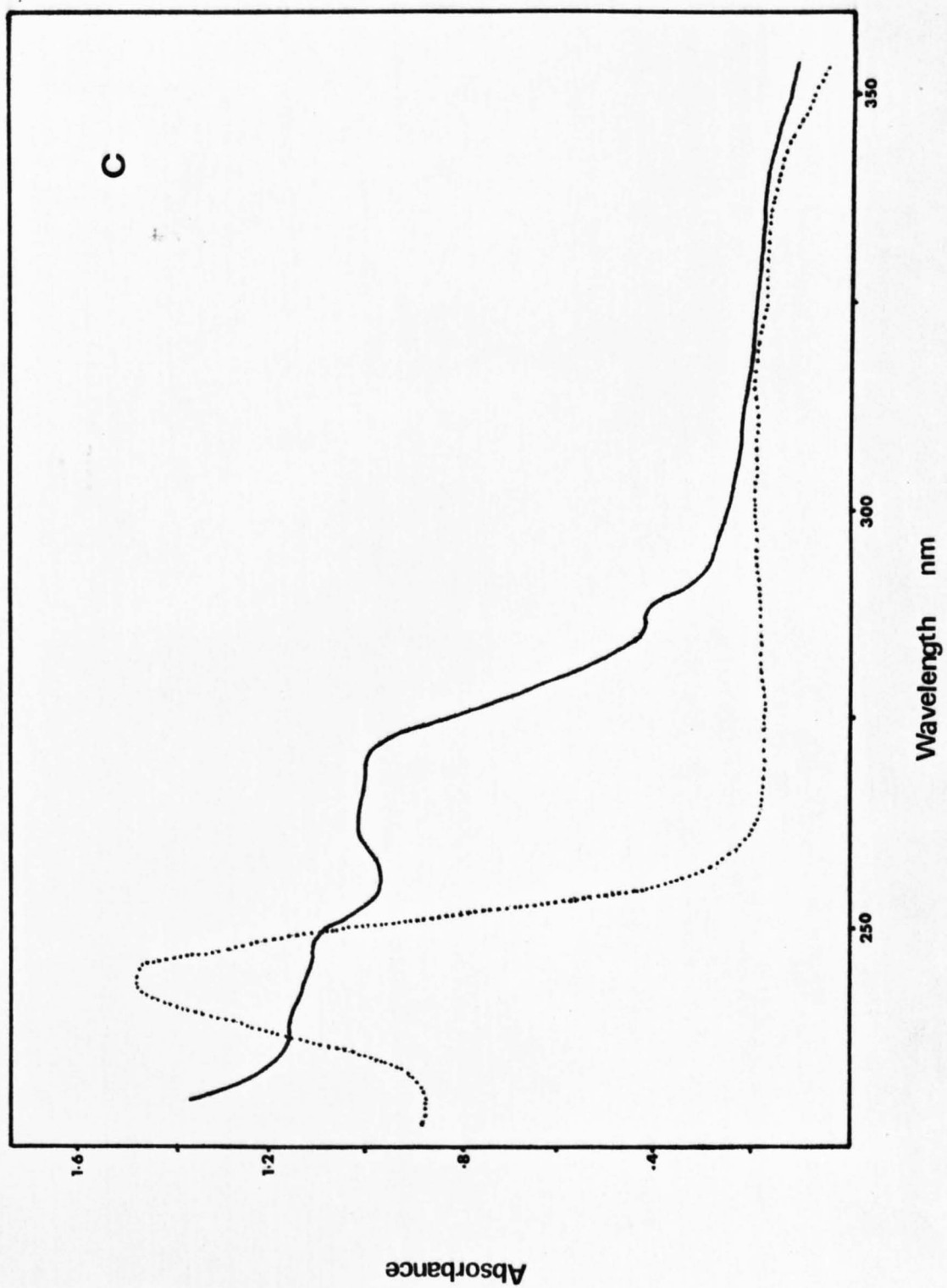
- oxidised, spectrum taken in methanol
- reduced with  $\text{NaBH}_4$  in methanol

(c) Strain 1M menaquinone containing fraction.

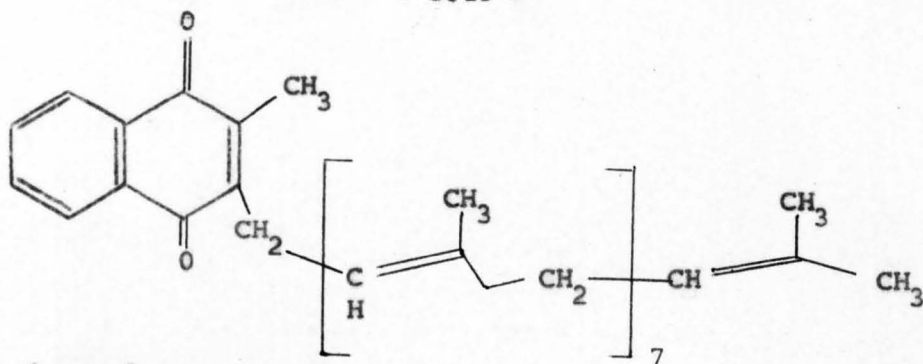
- oxidised spectrum in methanol
- reduced with  $\text{NaBH}_4$  in methanol







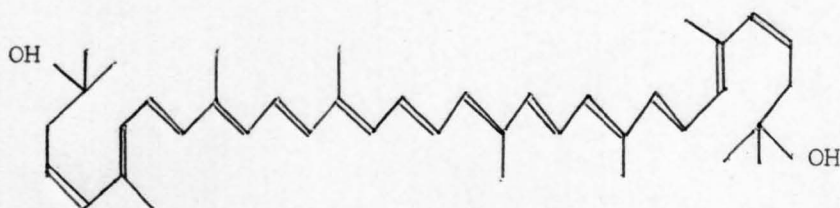




Menaquinone-8

The mutant menaquinone was readily reduced by  $\text{NaBH}_4$  in methanol and gave a spectrum essentially the same as wild-type menaquinone (Figure 5.6). However, the spectra in cyclohexane show that there is considerably less menaquinone in the mutant. The major peak at 249 nm is present in both strains, but the other peaks seen in strain 1 are not clearly defined in the mutant. Both fractions migrated to the same extent on T.L.C. using  $\text{CHCl}_3:\text{CH}_3\text{OH}$  or ether:light petroleum:acetic acid solvents.

The methylal fraction of strain 1 contained carotenoid. This has been shown to be a spirilloxanthin (Liaaen Jensen, 1962).



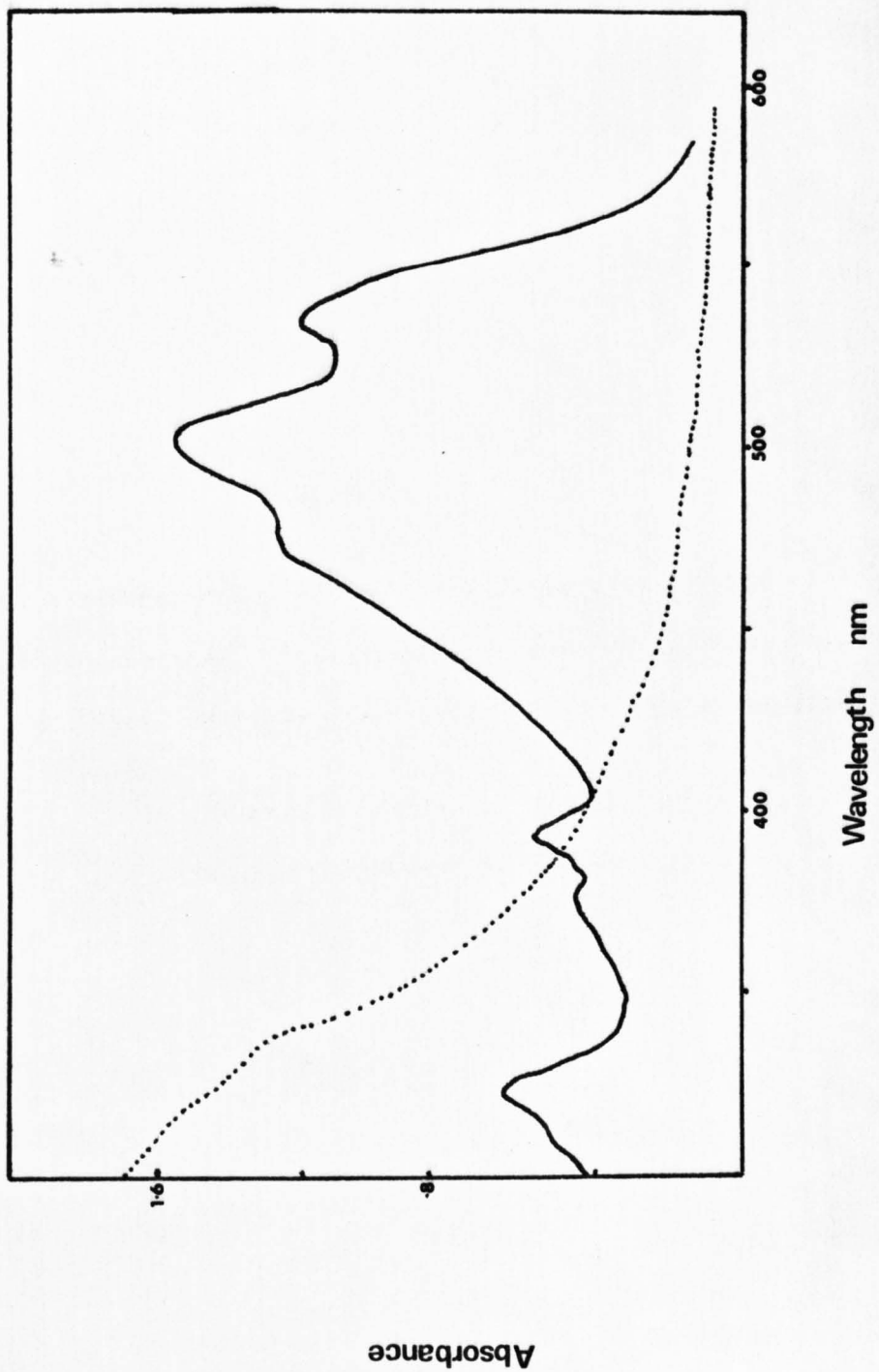
Bacterioruberin-α

Figure 5.6 shows the u.v. spectra of the methylal fractions from both strains. The typical triple peaked carotenoid absorptions

Figure 5.6

U.V. spectra of strain 1 and 1M lipids obtained in the methylal eluates from chromatography of neutral lipids on Decalso columns. The spectra were measured in  $\text{CHCl}_3$ .

—— Strain 1 (approx. 0.5 mg/ml)  
---- Strain 1M (approx. 1.0 mg/ml)



in the range 450 nm - 550 nm are completely absent from the mutant. The results from T.L.C. of the methylal fractions are shown in Figure 5.3. Before charring with 40%  $H_2SO_4$  two yellow spots were visible ahead of the red carotenoid spot in the wild-type lipid. These are possibly due to the decomposition of carotenoid since their relative amount increases in lipid extracted from late stationary phase cells. Also Brown (1969) reported that their concentration increased when dry lipid samples were heated for a short time. The level of carotenoid in the membranes was also shown to decrease as the culture aged (Brown and Stevenson, 1970). In the mutant a colourless component was present which corresponded to the carotenoid, but was preceded by only one yellow spot, although after charring a second component was visible on some extractions. These spots may have been due to the decomposition of a colourless carotenoid like compound. The amount of these two components increased in lipid from old cultures and the harvested cells from stationary cultures were markedly darker in colour.


#### 5.11 The extraction of lipid-free protein from the cell membrane

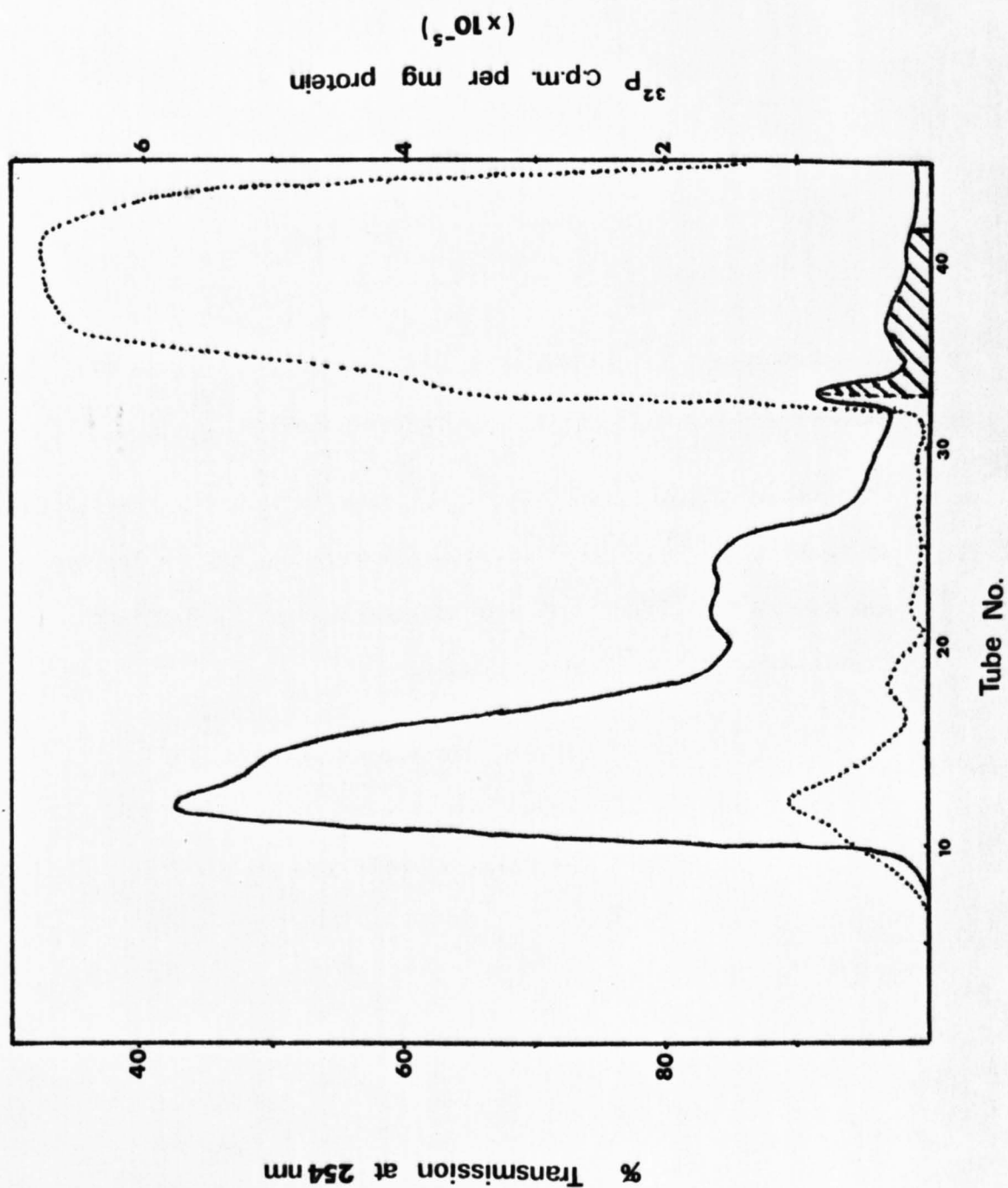
Membranes of strain 1 and 1M dissolved in 90% 2-chloroethanol in 0.01M HCl were separated into protein and lipid fractions by gel-filtration on Sephadex LH-20. Figure 5.7 shows the result obtained with strain 1 membrane which had been labelled with  $^{32}P$  inorganic phosphate. The mutant membrane gave a similar distribution

Figure 5.7

Gel-filtration of strain 1 membrane solubilised in 90% 2-chloroethanol in 0.01M HCl on Sephadex LH-20.

Experimental details are given in paragraph 5.4. Approximately 40 mg of solubilised membrane protein were applied to the top of the column and eluted with 90% 2-chloroethanol in 0.01M HCl. Fractions of 2.0 ml were collected.

----- c.p.m.  $^{32}\text{P}$  per mg protein ( $\times 10^{-3}$ )  
——— percentage transmission at 254 nm  
 carotenoid containing fractions



of protein and  $^{32}\text{P}$ . Tubes 9-30 and 34-43 were combined (i.e. protein-rich and lipid-rich fractions respectively) and extracted with  $\text{CHCl}_3:\text{CH}_3\text{OH}$  (2:1, v/v) to obtain lipid extracts. No lipid was detected in the protein-rich fractions, but a complete component of polar and non-polar lipids were obtained from the lipid-rich fractions when the extract was compared by T.L.C. with total lipid from untreated membranes.

The lipid-depleted protein was freed of 2-chloroethanol by extensive dialysis at  $4^\circ\text{C}$  against several changes of 10 volumes of  $0.01\text{M}$  Tris HCl pH 8.0 containing  $6\text{M}$  urea, 0.1% sodium dodecyl sulphate (SDS) and 0.1% 2-mercaptoethanol. Under these conditions the membrane protein remained soluble. When dialysis was carried out in the absence of urea, or at low pH, precipitation occurred after only a few hours of dialysis.

The solubilised protein was examined by polyacrylamide disc electrophoresis (see paragraph 5.6). Figure 5.8 shows the distribution of protein bands obtained with wild-type and mutant membrane protein. Both gels show essentially the same pattern with a predominant fairly slow moving band and several somewhat weaker bands. Strain 1M produced the same number of bands in the small-pore gel as the wild-type, although there was a slightly different distribution involving one fraction in the region of the main band. However, more material remained in the large-pore gel or at its surface in the case of strain 1M. The presence of

Figure 5.8

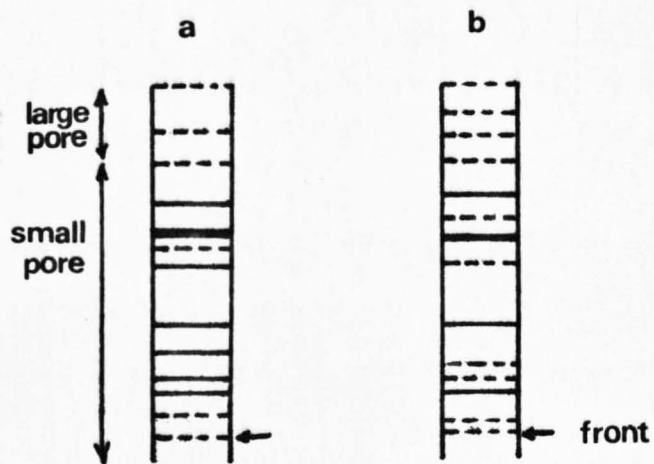
Polyacrylamide electrophoresis of lipid-free solubilised membrane protein.

See paragraph 5.6 for experimental details. Gels contained 5M urea and 0.1% SDS. Current was passed for 10 minutes at 3mA per gel and 2 hours at 5mA per gel. The gels were stained for protein with coomassie blue.

(a) strain 1

(b) strain 1M





lipoproteins was not detected when the material was stained with Sudan Black (see paragraph 4.5). The fact that considerably more material failed to move through the gel in the case of the mutant suggested that the proteins might differ to some extent not revealed by electrophoresis. In an attempt to resolve this possibility the preparations were analysed by gel-filtration and amino acid analysis.

Gel-filtration on Sephadex G100 and G200 in the presence of Tris HCl buffer containing 6M urea and 0.1% SDS failed to show any separation of proteins since the material was eluted from the column in more or less one peak in the void volume. This indicates that the protein was aggregated in particules of molecular weight greater than 200,000.

Amino acid analyses of strain 1 and 1M lipid-free protein are shown in Tables 5.3 and 5.4. The results are strikingly similar since only 3 residues show  $\Delta$ mole% values greater than 1.

Strain 1 lipid-free protein solubilised in 6M urea was subjected to isoelectric focussing by a slightly modified version of the technique described in paragraph 3.3. In order to keep the membrane protein solubilised the sucrose-Ampholine solution contained 6M urea. Prior to the focussing run the solubilised protein was desalted by dialysis for 20 hours at 4°C against 3 changes of 5mM Tris HCl pH 8.0 containing 6M urea. Otherwise

Mole per cent

	lipid-free membrane protein		SP detergent-(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> preparation	
	Strain 1	Strain 1M	Strain 1	Strain 1M
lys	2.1	2.0	3.1	3.0
his	1.1	1.2	1.6	1.7
arg	2.9	4.2	5.1	5.0
asp	11.4	11.4	9.0	8.8
thr	8.5	10.1	5.8	6.0
ser	6.7	7.1	6.5	6.5
glu	9.9	10.6	6.6	8.0
pro	4.1	3.9	5.8	3.1
gly	11.2	11.5	8.8	8.8
ala	10.7	9.1	12.0	10.2
$\frac{1}{2}$ -cys	0.3	0	0	0
val	8.9	8.7	6.9	6.8
met	1.6	1.4	2.5	2.7
ileu	4.3	4.3	5.1	5.0
leu	8.7	8.6	12.8	13.0
tyr	2.4	2.4	3.1	3.0
phe	3.5	3.6	5.1	5.3

mole per cent = moles of amino acid per 100 moles of total amino acids

Table 5.3 Amino acid analyses of lipid-free membrane protein  
and SP prepared by the detergent/-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> method.

the experiment was carried out as described in Chapter III.

Figure 5.9 shows the distribution of material in the column when it was emptied after 48 hours. The first few fractions were rather turbid, hence the large drop in transmission registered by the Uvicord for the first 10-15 ml. At the bottom of the column the proximity of the acid anode solution probably caused the precipitation of some protein at the beginning of the run. There was also a tendency for proteins to precipitate after they had migrated to a position in the column where the pH corresponded to their isoelectric points. For this reason protein loadings were kept low (5-10 mg).

The separation of protein components shown in Figure 5.9 indicates that the membrane proteins are of an acidic character since the bulk of the sample protein was recovered within the pH range 3-7.

#### 5.12 Extraction of structural protein from purified membranes

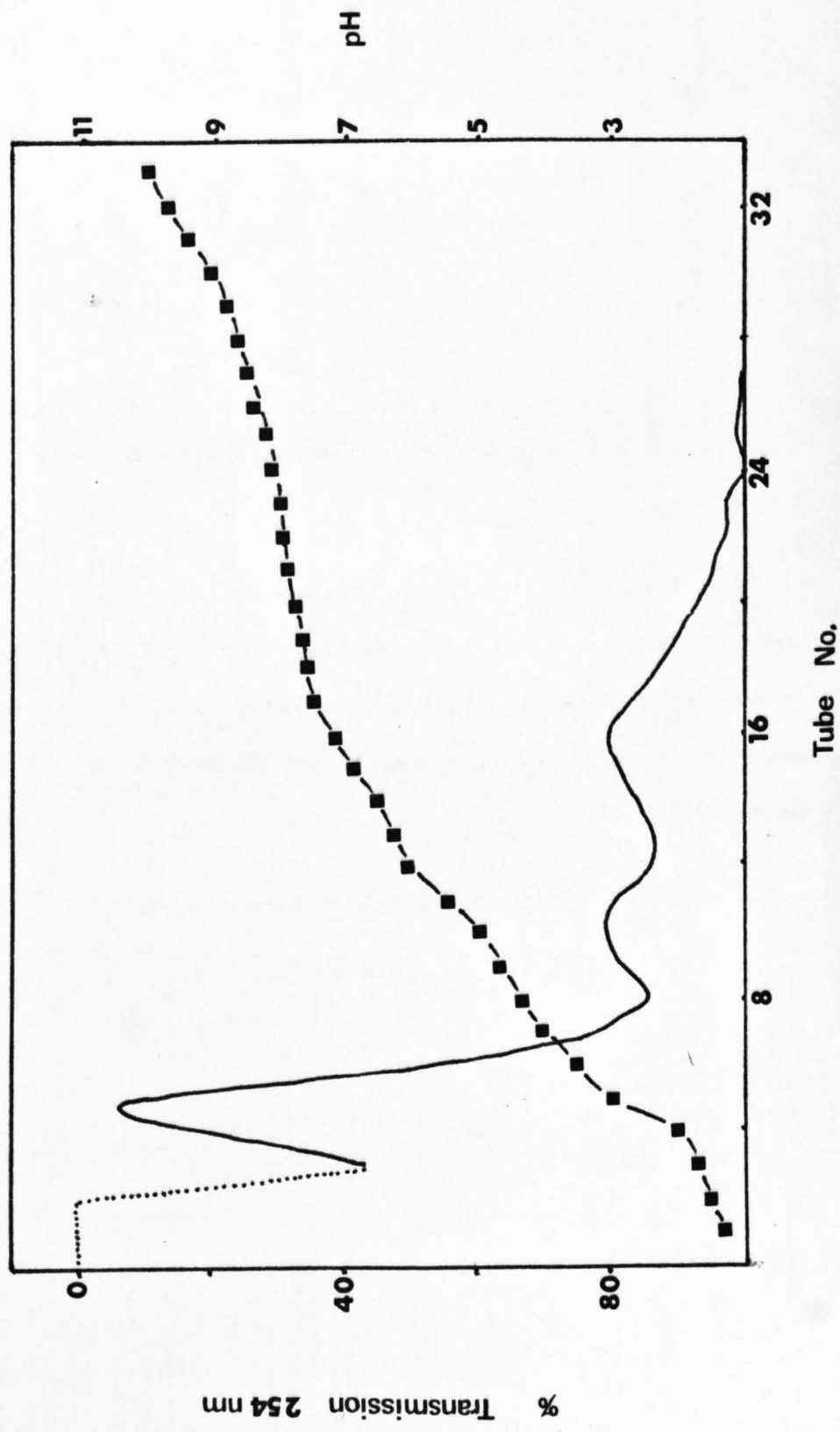
Structural proteins (SP) were isolated from purified membranes of strains 1 and 1M by the methods described in paragraph 5.5. Both methods of extraction gave preparations coloured slightly "off white". SP extracted with mixed detergents/ $(\text{NH}_4)_2\text{SO}_4$  constituted 20% of the original membrane protein whilst the acid extractable protein gave yields varying between 15% to 30%. The

Figure 5.9

Isoelectric focussing of lipid-free membrane protein from strain 1.

The isoelectric focussing column contained a sucrose - Ampholine -6M urea gradient. Current was passed for 48 hours to develop a pH 3-10 gradient. The contents of the column were then removed and collected in 3.0 ml fractions. (See paragraph 5.13).

—— Percentage transmission 254 nm  
■ pH



mutant membrane gave similar yields of SP as strain 1. Both types of preparations were completely insoluble in the Tris HCl buffer at neutral pH. No cytochromes or carotenoids were detected in SP prepared by either procedure.

SP samples were characterised by polyacrylamide disc electrophoresis and the detergent preparation also by amino acid analysis. Diagrams of gels are shown in Figure 5.10 and amino acid analysis results in Table 5.3.

Electrophoresis revealed that SP preparations by either procedure were rather heterogeneous and somewhat variable in content when different preparations were compared. The mutant gave essentially the same results as strain 1. In SP prepared by the detergent method, a slow moving protein was always present in much greater concentration than the other bands. In all the preparations tested a considerable amount of material remained at the origin. This effect has been reported previously with mitochondrial SP (Lenaz et al, 1968). In that case material which remained at the origin was shown after performic acid treatment to give similar bands as the original material.

PGE revealed that the detergent method gave SP of a more consistently reproducible content than the acid extraction procedure, therefore amino acid analysis was only carried out on the former. A comparison of amino acid analysis results is shown in Table 5.4.

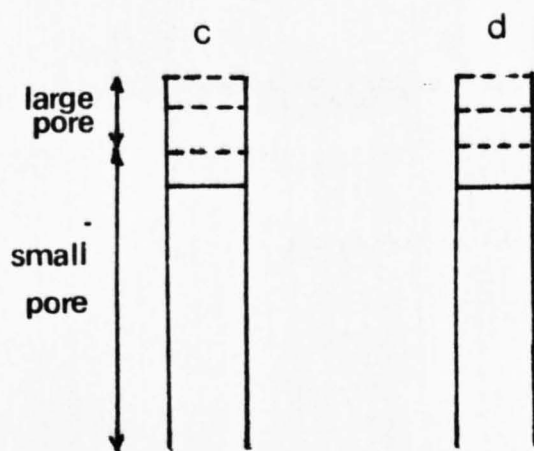
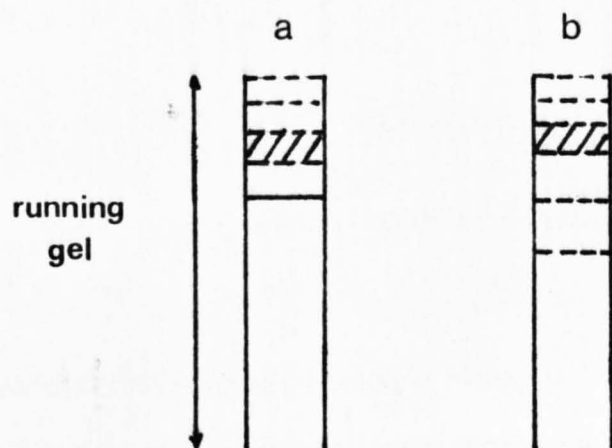
Figure 5.10

Polyacrylamide disc electrophoresis of SP preparations from strain 1 and 1M.

SP was prepared from purified membrane by acid extraction or detergent  $-(\text{NH}_4)_2\text{SO}_4$  precipitation techniques (see paragraph 5.5). Details of P.G.E. methods are given in paragraph 5.6. Gels were stained for protein with coomassie blue.

- |               |   |  |
|---------------|---|--|
| (a) strain 1  | } | detergent $-(\text{NH}_4)_2\text{SO}_4$ extraction |
| (b) strain 1M |   |  |
| (c) strain 1  | } | 0.1N HCl extraction                                |
| (d) strain 1M |   |  |





Sample	Standard	No of amino acid residues		
		$\Delta$ mole% < 1	> 1	> 2
lipid-free membrane protein (strain 1M)	lipid-free membrane protein (strain 1)	14	3	0
SP detergent (strain 1M)	SP detergent (strain 1)	14	2	1
Beef heart MSP <sup>a</sup>	"	11	1	5
Yeast MSP <sup>a</sup>	"	10	1	6
<u>Neurospora crassa</u> MSP <sup>a</sup>	"	10	4	3
Erythrocyte membrane SP <sup>b</sup>	"	11	3	2
<u>Hydrogenmonas facilis</u> membrane SP <sup>c</sup>	"	8	4	5
Lipid-free membrane protein (strain 1)	"	7	4	6

Table 5.4 Comparison of amino acid analyses of lipid-free membrane protein and SP from various sources.

$\Delta$  mole% = mole% of residues of standard - mole% of corresponding residues of sample proteins.

<sup>a</sup> Criddle (1969)

<sup>b</sup> Schneiderman and Junga (1968)

<sup>c</sup> Kuehn et al (1969)

MSP = mitochondrial structural protein

SP preparations of strain 1 and 1M have a similar amino acid content, only 3 residues having mole% values differing by greater than 1 mole%. Strain 1 SP is also compared to total membrane protein and SP preparations from other organisms.

### Section III : Discussion

Proteins together with lipids account for the bulk of the membrane dry weight. However, ratios of protein and lipid are far from identical in different membranes, sterol is not always present and the nature of the polar lipids can vary widely, (Korn, 1969a). The membranes of halophilic bacteria contain a higher proportion of lipid than other bacteria investigated. Gram-positive bacteria tend to have protein to lipid ratios within the range 2.0-4.0 (Reavely, 1968; Korn, 1969b). The only reliable results of Gram-negative bacteria were obtained with Mycoplasma species. These organisms are devoid of cell walls and intracytoplasmic membranes and cell preparations of high purity have been obtained by osmotic lysis (Razin, 1969). They contained 50-60% protein and 30-40% lipid (Razin, 1967; Smith, 1967). The results in paragraph 5.9 indicate a 1:1 ratio of protein to lipid in H. salinarium membranes.

This contrasts somewhat with the result obtained by Kates et al (1965) who reported a ratio of 1:8 in samples from H. cutirubrum. Previously high lipid contents have been reported in membranes from a wide range of eucaryotic sources e.g. chloroplast lamellae, (Lichtenhaler et al, 1963), myelin (Morrocks, 1967), and mitochondrial outer membranes (Allmann et al, 1968; Newman et al, 1968).

The phospholipids of H. salinarium show basically a similar distribution on T.L.C. to those of H. cutirubrum, although the colourless mutant of H. salinarium has two additional components i.e. spots 4 and 5 (see Figure 5.1). Spot 4, the most prominent component of the mutant phospholipid did not incorporate  $\text{[2-}^{14}\text{C]}$  mevalonate, unlike the major constituents of H. cutirubrum and strain 1 (spot 6), which both incorporated  $\text{[2-}^{14}\text{C]}$  mevalonate and probably represent the structure suggested by Kates et al (1965) (see paragraph 5.10).

Differences between mutant and wild type were also detected in the non-polar lipid fractions. The colourless mutant lacked the red carotenoid, bacterioruberin  $\alpha$ , although on T.L.C. a colourless component was detected in a similar position as the red constituent of strain 1 neutral lipid. No significant qualitative differences were detected in the neutral lipid fractions although menaquinone was present at a much lower concentration in the mutant extracts. This is of interest because menaquinone and carotenoid are biosynthetically related (Richardson and Hendrickson, 1964) both involving the synthesis and condensation of isoprenoid units.

Solubilised protein was obtained from purified H. salinarium membrane by a method which combined organic solvent extraction and gel-filtration. The results reported in paragraph 5.11

indicate that this preparation produces protein which is essentially free of membrane lipid and which can be readily solubilised in aqueous concentrated urea solutions. An alternative procedure utilising a simple  $\text{CHCl}_3:\text{CH}_3\text{OH}$  (2:1 v/v) extraction caused protein to immediately precipitate as fairly large particles which were difficult to break up and remained insoluble after prolonged exposure to detergents and urea. However, the solubilised preparation yielded protein which was successfully examined by gel-filtration, PGE, isoelectric focussing and amino acid analysis.

In general the results are characteristic of membrane proteins obtained from other sources. In aqueous solution in the absence of any disaggregating agents the protein tends to remain insoluble and even when solubilised, as indicated by gel-filtration, there is a marked tendency for aggregation into particles of very high M.W. to occur (Morgan and Hanahan, 1966). Amino acid analysis of strain 1 and 1M lipid-free membrane protein revealed a close similarity. However, PGE showed, perhaps not unexpectedly, that the preparations were heterogeneous and could be separated into at least 13 bands. In the case of strain 1 preparations all of this material was shown to be of an acidic nature by isoelectric focussing. The bulk of the protein recovered having pI values within the range 3.0-7.0. This result seems typical of halophilic proteins, since purified MDH (see Chapter III) had a pI of 5.0-6.0 and previously Brown (1965) and Larsen (1967) have postulated an

overall acidic character for halophilic envelope proteins.

About 20-30% of the membrane protein from either strain could be isolated by the procedure previously used for the preparation of SP from mitochondrial membranes. More consistent results were obtained with the detergent  $(\text{NH}_4)_2\text{SO}_4$  method of Criddle than with an acid extraction procedure. Amino acid analysis of SP from wild-type and mutant revealed a close similarity between the strains and in general an overall similarity with SP from other sources e.g. mitochondria and erythrocytes (see Table 5.4). Previously SP has been characterised by its lack of solubility in aqueous solvents at neutral pH and ability to bind phospholipids. Amino acid analyses have tended to reveal a predominance of apolar amino acids. Thus the ratio H/A (see Discussion Chapter IV) would be expected to be low in the case of SP,

In Table 5.5 the H/A values of various membrane and non-membrane protein are compared. The SP preparations of H. salinarium are particularly apolar in a similar category to myelin proteolipid. This especially is noteworthy, since myelin, like Halobacterial membrane has a particularly high content of lipid.

In Chapter IV it was shown that the residue remaining after urea treatment, representing about 60% of the membrane protein gave an H/A of 1.2 i.e. similar to the SP preparation. Thus a high

Source	H/A	Reference
<u>H. salinarium:</u>		
Strain 1 lipid free membrane protein	1·6	
Strain 1M " " " "	1·8	
Strain 1 SP	1·2	
Strain 1M SP	1·2	
Mitochondrial SP Beef Heart	1·5	} a
" Yeast	1·7	
" Neurospora	1·6	
Human erythrocyte SP	1·3	b
" " lipoprotein	1·6	c
Neurospora membrane SP	1·9	} d
<u>Hydrogenomonas facilis</u> SP	1·8	
Myelin proteolipid	1·0	e
Flagellin <u>B. subtilis</u>	1·9	} f
" <u>Spirillum serpens</u>	2·3	
Bovine plasma albumin	2·1	g
Halophilic bacterial bulk protein	1·9	h

Table 5.5 Apolar character of membrane and non-membrane proteins isolated from various sources.

References (a) Criddle (1969); (b) Schneiderman and Junge (1968); (c) Morgan and Hannahan (1966); (d) Kuehn et al (1969); (e) Tenenbaum and Folch (1966); (f) Martinez et al (1967); (g) Hatch and Bruce (1968); (h) Reistad (1970).



proportion of Halobacterial membrane protein is very hydrophobic in character, more so than SP or membrane protein from various other courses. Conversely certain fractions released from the membrane by urea treatment are very polar. At this stage it is impossible to relate the significance of these findings to halophilism but previously Brown (1964) has shown that by chemically increasing the polarity of the outer wall proteins of a marine bacterium the halophilic character of the cell could be increased. Thus possibly the hydrophobic protein and lipid constitute a highly apolar lipoprotein matrix with highly polar protein which can be relatively easily removed residing on the exterior of the membrane.

## Chapter VI : The effect of detergents on the cell membrane

### Introduction

The problem of assembly of cellular membranes is usually approached on the assumption that lipid-lipid and lipid-protein interactions are the main stabilising feature of the membrane structure. It is possible, however, that large regions of cellular membranes are comprised of protein or lipoprotein lattices with major cohesive forces arising between proteins (Wallach and Gordon, 1968; Green and Perdue, 1966; Green, 1968; Changeux et al, 1967). The associations between membrane lipids and proteins and between the proteins themselves must be through weak interaction, i.e. ionic, hydrogen bonding, London-Van der Waals forces and/or hydrophobic bonding (Wallach, 1969) rather than covalent linkages since the lipids of cellular membranes may be separated from proteins under mild conditions by organic solvents or detergents. In addition, there is evidence that the interactions between certain membrane proteins can also be disrupted by the action of detergents (Engelman et al, 1967; Green and Fleischer, 1967; Salton, 1967a, 1968).

In the traditional models of membrane structure the forces binding proteins to lipids are considered primarily ionic (Danielli and Dawson, 1935; Robertson, 1959). However, there is no general evidence that ion pairing is responsible for the strong lipid-protein associations of cell membranes. For example, the

acidic phosphatides of H. halobium cannot be released from their membrane protein complexes by variations of ionic strength or pH (Brown, 1965) although these changes may cause the disruption of artificial lipid membranes (Salem, 1962; van Deenen, 1965).

Phosphatides can, however, usually be extracted from cell membranes by  $\text{CHCl}_3:\text{CH}_3\text{OH}$  (2:1), a solvent which would be expected to stabilise ionic interactions. Thus the effectiveness of organic solvents and detergents in the extraction of lipids and solubilisation of proteins provides strong evidence for the importance of hydrophobic bonding between these membrane components.

In Chapter IV it was shown that although a certain proportion of protein could be disaggregated from the membrane of H. salinarium by urea, the bulk of lipid and protein remained as a lipoprotein complex of very high M.W. The membrane lipid and protein were, however, successfully extracted and purified by the use of organic solvents as described in Chapter V.

In the present chapter, experiments are described in which the interactions of membrane lipids and proteins were investigated. Purified membrane preparations were disaggregated with detergents and the material released fractionated by gel-filtration and P.G.E. The major fractions obtained were characterised by analysis of their components in an attempt to distinguish important differences which might have bearing on the manner in which they interact in the membrane.

## Section I : Methods

6.1 Centrifugation on sucrose gradients: 0-40% linear sucrose gradients were prepared and centrifuged in the manner described in paragraph 4.4. Discontinuous gradients for centrifugation at 160,000 x g in an SW 50L rotor of a Spinco ultracentrifuge were prepared by carefully layering 1 ml aliquots of 10%, 20%, 30% and 40% sucrose in 0.01M Tris HCl pH 8.0 into a centrifuge tube. Finally the protein solution, 5-10 mg in 1.0 ml of the same buffer was layered on to the top of the gradient.

6.2 Gel-filtration: Gel-filtration on columns of Sepahdex G100 or Agarose A50m was carried out using the apparatus described in paragraphs 4.3 and 4.6. The buffers used were as described in the experimental results of Section II.

6.3 Polyacrylamide gel electrophoresis (P.G.E.): The technique employed was that described in paragraph 4.5 modified by the use of different buffers, i.e. presence of SDS or urea as indicated in the results section of this chapter.

6.4 Assay of protein, lipid, cytochrome and carotenoid: Routine quantitative assays of protein, lipid, carotenoid and cytochrome were made by the procedures described in paragraphs 2.5, 5.1, 4.7 and 4.8 respectively.

Qualitative analysis of cytochrome by measurements of oxidised

versus reduced difference spectra were carried out using a Bristol split-beam spectrophotometer operated by W.E. Lancashire.

This instrument is especially suitable for biological material because the photomultiplier is close to the cuvette holder and allows accurate spectra to be determined for turbid solutions in which scatter becomes an important source of error. The instrument also has a useful sensitivity i.e. o.d. ranges 0-0.5 up to 0.1.0.

## Section II : Results

### 6.5 Solubilisation of membrane with nonionic or ionic detergents

Dilute suspensions of strain 1 or mutant membrane were visibly clarified on the addition of nonionic (Triton X - 100) or anionic (SDS) detergent. The extent of solubilisation was estimated by measuring changes of turbidity after the addition of different amounts of detergent. Aliquots of purified membrane containing 10 mg of protein in 0.1M Tris HCl pH 8.0 were treated with Triton X - 100 or SDS, incubated at room temperature for 15 minutes, diluted to 3.0 ml with water and the turbidity of the final solution measured with an EEL colourimeter. The results of an experiment using mutant membrane are shown in Figure 6.1. Similar results were obtained with strain 1 membrane. Both types of detergent clarify the membrane suspensions to about 10% of the original turbidity. However, SDS is more effective than Triton.

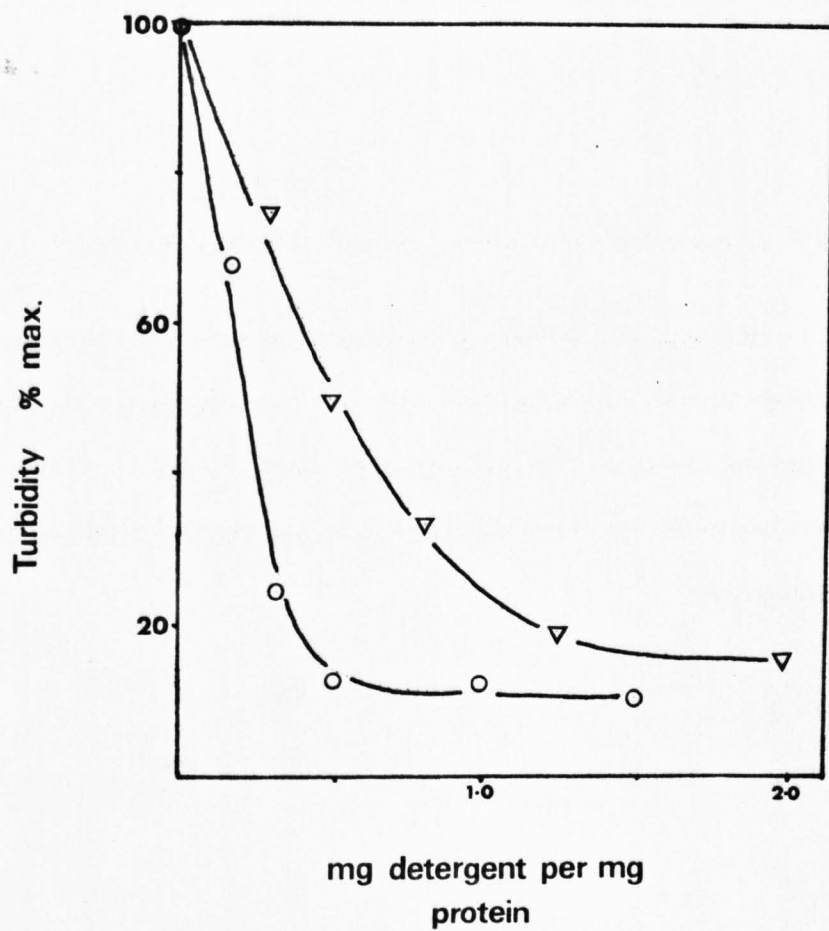
Figure 6.1

The solubilisation of strain 1M membrane with detergents

Suspensions of purified membrane were titrated with SDS or Triton X-100. The turbidities of the resulting solutions were measured with an EEL colorimeter (608 filter) zeroed for each reading with buffer containing an appropriate concentration of detergent.

○ SDS

▽ Triton X-100



Treatment with 1.0 mg of SDS/mg of protein reduces turbidity by 90%, while 2.0 mg Triton X - 100/mg protein reduces turbidity by 85%.

A solution of mixed anionic detergents (MAD, see paragraph 5.5) was also very effective in solubilising membrane suspensions. When 0.2 ml of MAD was added per 10 mg membrane protein, a 90% drop of turbidity was detected.

#### 6.6 Characterisation of membrane solubilised with ionic or non-ionic detergents

Samples of membrane treated with SDS, MAD or Triton to give maximum clarification were not sedimented when centrifuged at 150,000 x g for 4 hours in a Spinco ultracentrifuge at 4°C. The resulting supernatants were used for the characterisation of the disaggregated membrane particles by sucrose gradient centrifugation gel-filtration, polyacrylamide gel electrophoresis (P.G.E.) and chemical analysis.

Sucrose gradient centrifugation: Untreated purified strain 1 membrane and membranes solubilised with SDS or Triton were centrifuged on linear sucrose gradients of 0-40% sucrose at 60,000 x g for 2½ hours as described in paragraph 4.4. Figure 6.2 shows the distribution of material in the fractions collected when the tubes were emptied. The detergent treated membranes remained at the top of the gradients. The untreated membrane behaved as



Figure 6.2

Sucrose density gradient centrifugation of  
strain 1 membrane treated with detergents

Samples of untreated and detergent solubilised membranes  
were centrifuged at  $60,000 \times g$  for  $2\frac{1}{2}$  hours on linear sucrose  
gradients of 0-40% (see paragraph 4.4).

(a) Untreated purified membrane ———


Fraction F1 - - - -

(b) Purified membrane treated with SDS

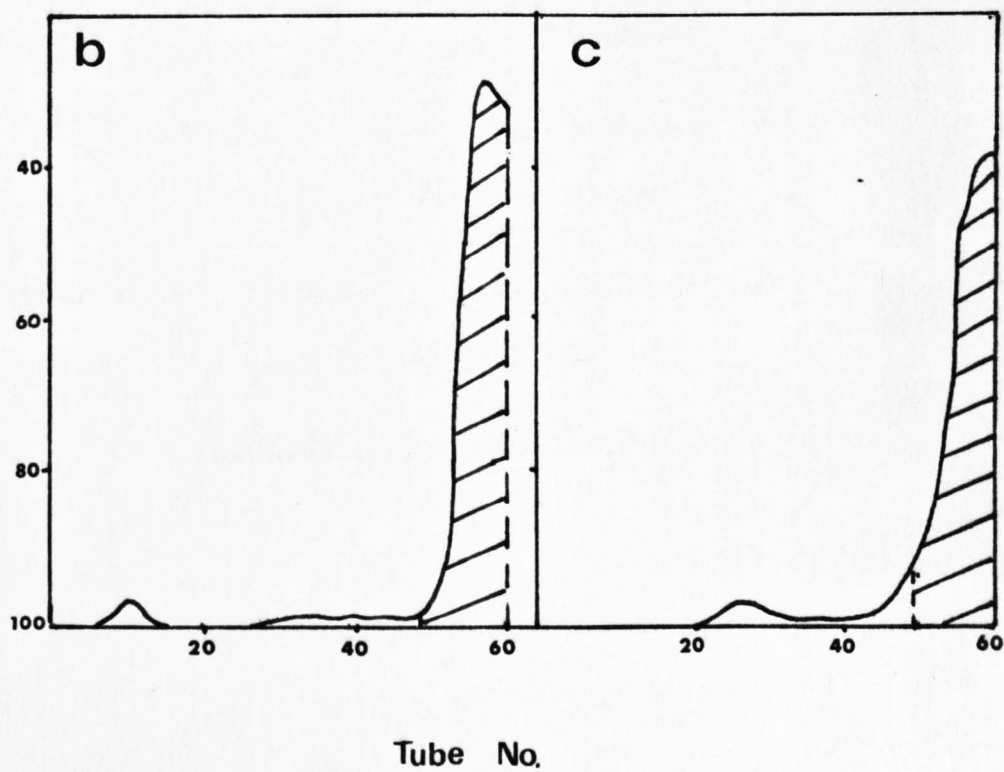
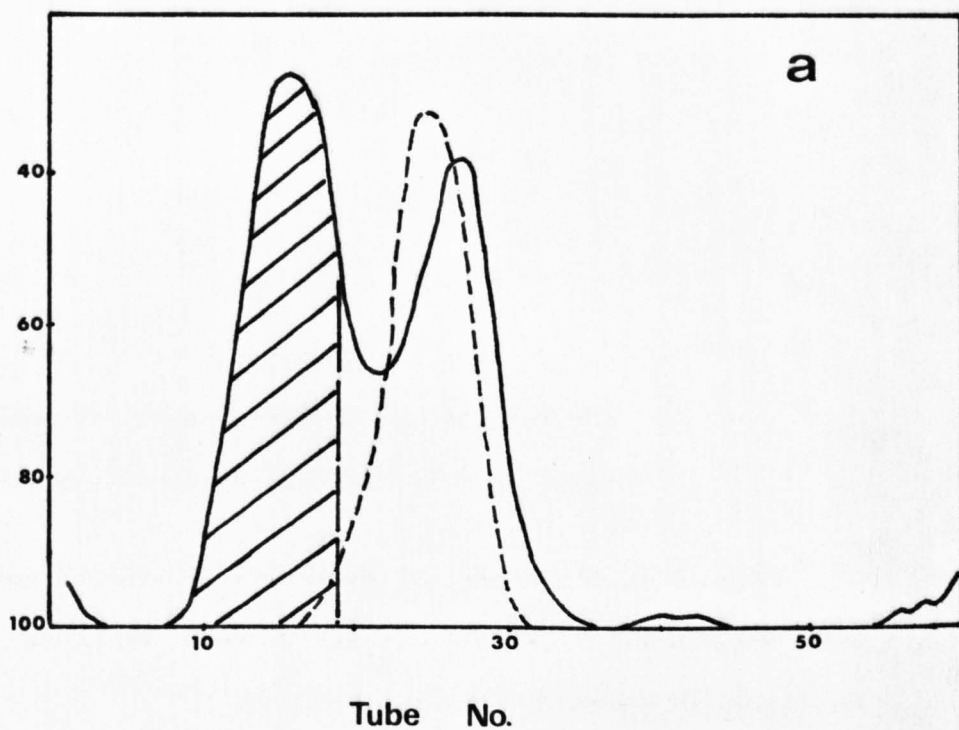
(1 mg SDS/mg protein)

(c) Purified membrane treated with Triton X-100

(2 mg X-100/mg protein)

Fractions containing carotenoid 

Percentage Transmission at 254nm



previously noted (see paragraph 4.13) forming a well defined band containing approximately 80% of the protein, and all of the carotenoid, followed closely by a smaller colourless band. The experiment was repeated using strain 1 membrane on discontinuous gradients (see paragraph 6.1) centrifuged at  $160,000 \times g$  for 4 hours. The detergent treated membrane still remained at the top of the gradient while the untreated membrane produced a sharp red band about half-way down the tube.

Gel-filtration on Sephadex G100: Whitaker (1963) and Andrews (1964) have demonstrated the use of Sephadex gel-filtration as a method of protein molecular weight determination. Whitaker showed that the logarithm of the molecular weight of a protein bears a linear relationship with the ratio of the elution volume ( $V_e$ ) to the column void volume ( $V_o$ ). A 1.5 cm G100 column previously calibrated with a series of standard proteins (see paragraph 6.2 and Figure 6.3) was used to estimate the molecular weight of the particles released from strain 1 membrane by SDS treatment. Prior to the application of the membrane sample the column was equilibrated with  $0.01M$  Tris HCl pH 8.0 containing  $0.1M$  NaCl and 0.5% SDS. Under these conditions the membrane material was eluted as a single peak with a  $V_e/V_o = 1.48$  indicating by reference to Figure 6.3 a molecular weight of 130,000. Since the effect of SDS on each of the standards was not known the column was calibrated in the absence of SDS. Previously Rosenberg and

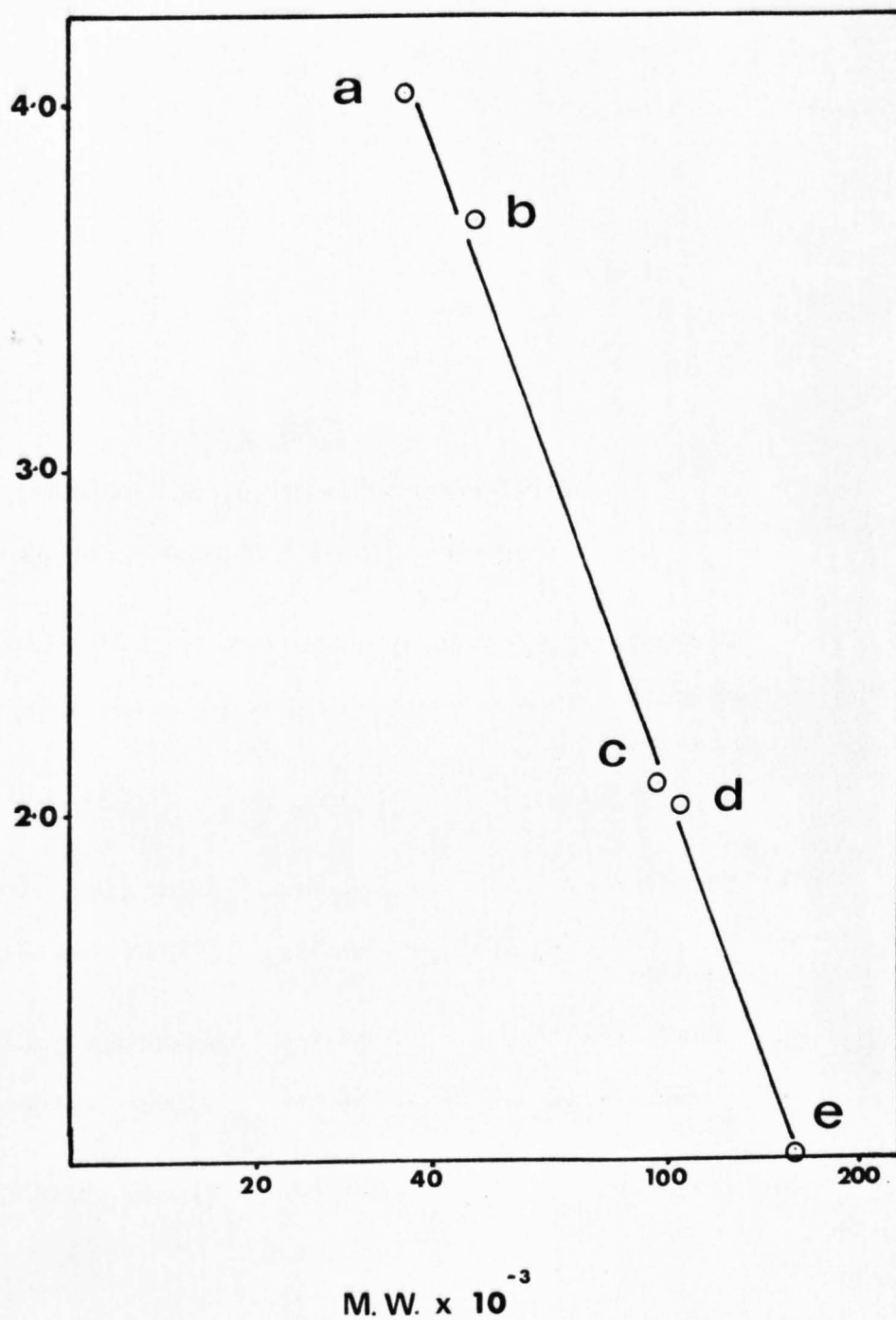
Figure 6.3

Relationship between M.W. and relative elution  
volume ( $\frac{V_e}{V_o}$ ) for a Sephadex G100 column

A series of proteins of known M.W. were eluted from the  
column under standard conditions (see paragraph 6.2).

<u>Protein</u>	<u>M.W.</u>	<u>Reference</u>
(a) Pepsin	35,000	Bovey et al (1960)
(b) $\alpha$ Amylase ( <u>B. subtilis</u> ) monomer	48,000	Fischer and Stein (1960)
(c) Yeast hexokinase	96,000	Macdonald (1960)
(d) $\alpha$ Amylase ( <u>B. subtilis</u> ) dimer	96,900	Fischer and Stein (1960)
(e) Aldolase	160,000	Kawahara and Tanford (1966)

$$\frac{V_e}{V_o}$$



Guidotti (1962) have reported that the pore size of some gel-filtration resins is altered in the presence of unbuffered SDS. Thus it is possible that the  $\frac{V_e}{V_o}$  value determined for the solubilised membrane in the presence of SDS may not be directly comparable to the calibration curve determined in the absence of SDS.

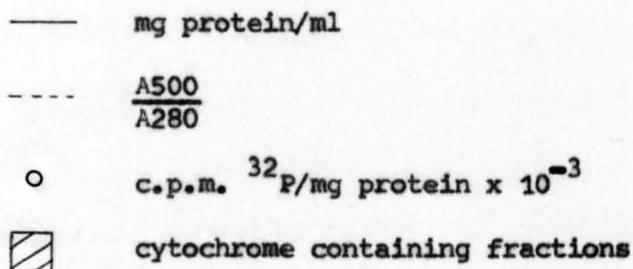
When SDS solubilised membrane was eluted from G100 with SDS-free Tris HCl buffer all of the membrane protein and carotenoid was eluted as a single peak in the column void volume. Figure 6.4 (a) shows the distribution of protein and carotenoid in the column effluent. However, MAD solubilised membrane was separated into protein-rich and lipid-rich fractions by gel-filtration on G100. Figure 6.4 (b) shows the distribution of material in the column effluent when MAD solubilised strain 1 membrane labelled with  $^{32}\text{P}$  was eluted with detergent-free Tris HCl buffer. Strain 1M membrane solubilised with MAD gave a similar distribution of protein and  $^{32}\text{P}$ . At critical concentrations detergents may form micelles of high molecular weight (Kushner and Hubbard, 1954). Therefore, to determine the position of elution of detergent micelles a control was run of MAD at the same dilution as in the MAD-membrane samples. Figure 6.5 shows the distribution of detergent in the effluent. A proportion of the detergent was eluted in a position indicating a M.W. of about 120,000 while the remainder was of low molecular weight, i.e. less than 4,000.

Figure 6.4

Gel-filtration of detergent solubilised  
strain 1 membrane on Sephadex G100

Samples of purified membrane solubilised with SDS or MAD were applied to 1.5 x 55 cm columns of G100 and eluted with detergent free 0.01M Tris HCl pH 8.0 containing 0.1M NaCl.

- (a) Purified membrane solubilised with SDS, (1 mg/mg protein)
- (b) Purified membrane labelled with  $^{32}\text{P}$  and solubilised with MAD, (0.2 ml/10 mg protein, see paragraph 5.5 for composition of MAD).





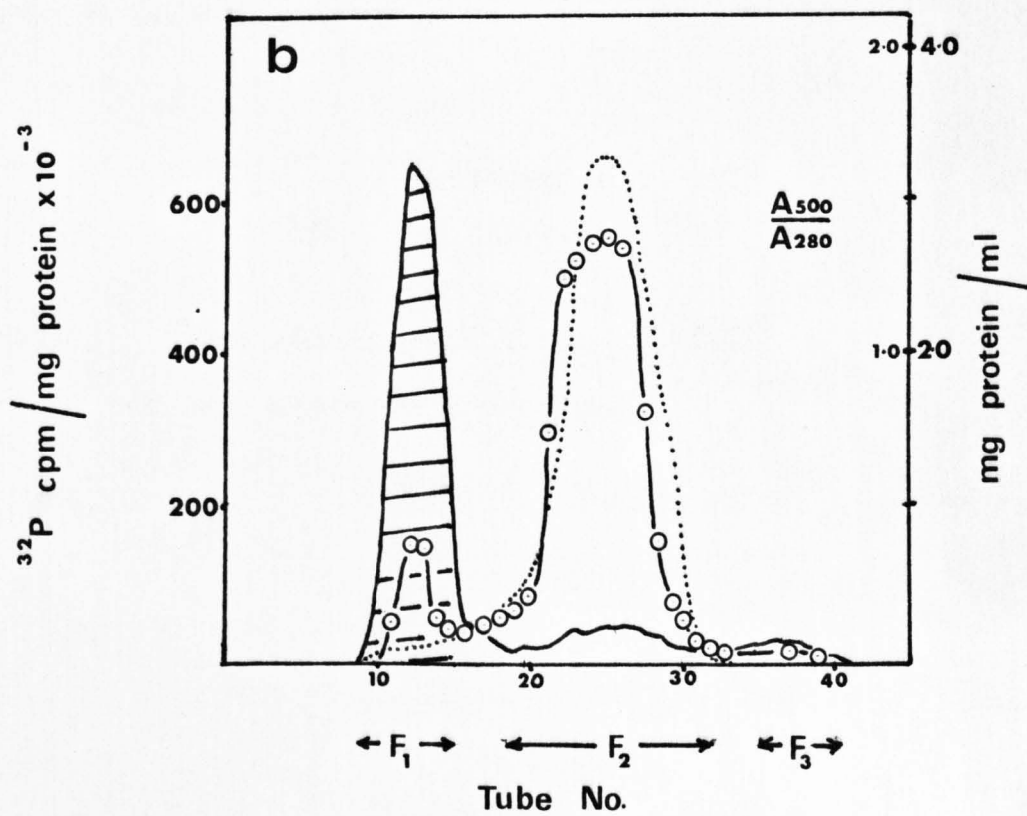
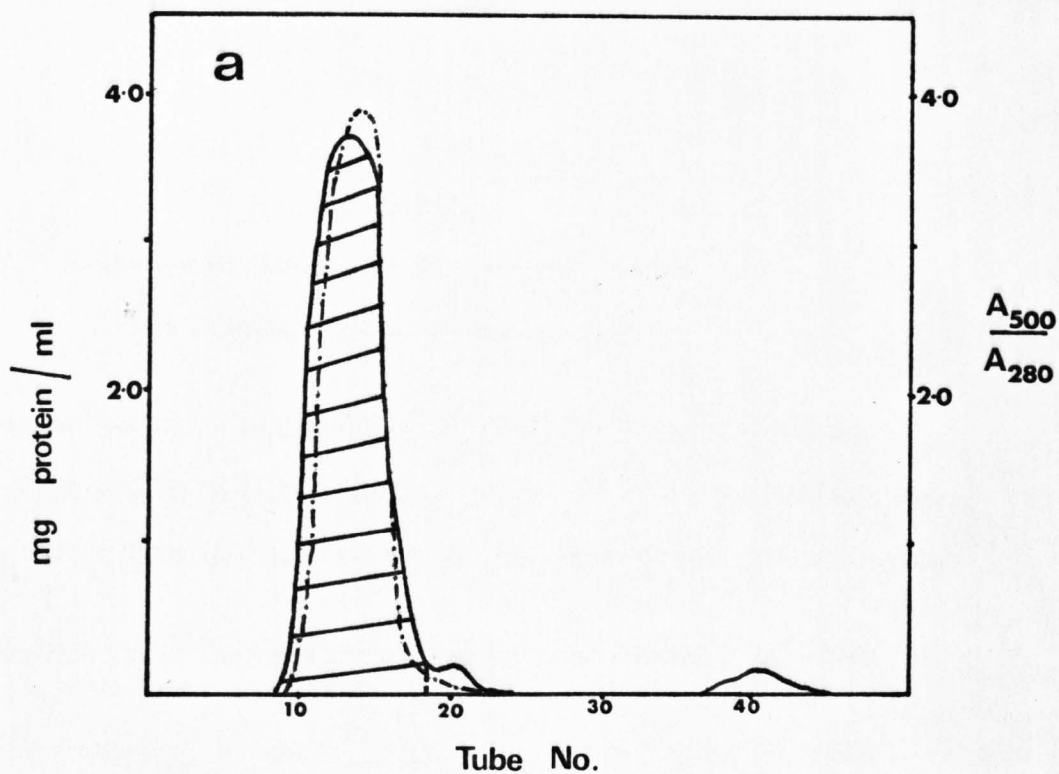


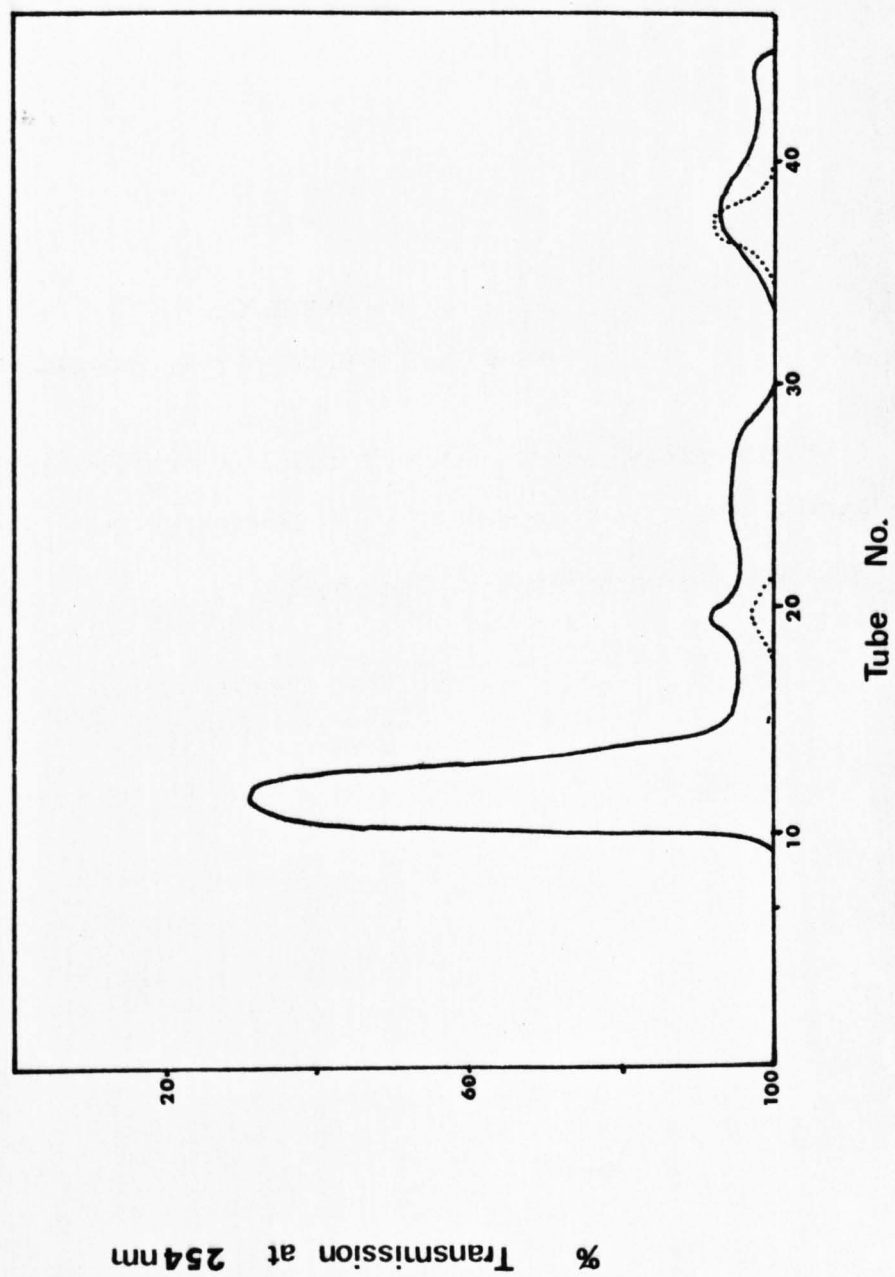


Figure 6.5

Gel-filtration of MAD on Sephadex G100

A sample of MAD at the same dilution as in membrane-MAD samples was eluted from a 1.5 x 55 cm G100 column with 0.01M Tris HCl pH 8.0 containing 0.1M NaCl.

—— Purified membrane + MAD  
---- MAD alone



Peaks corresponding to detergent were clearly visible in the MAD-membrane traces.

The tube contents from the G100 effluent were combined as indicated in Figure 6.4 (b) to give three fractions:

F1 - a brown solution containing material of high M.W.

F2 - a red solution (colourless in the case of strain 1M) containing a lipoprotein complex of M.W. approximately 80,000 - 90,000.

F3 - a colourless low M.W. fraction corresponding to detergent.

The high molecular weight detergent fraction was avoided, but traces may have been present in both F1 or F2. Thus these fractions were further purified separately by concentrating on an ultrafilter and reapplying to the G100 column in the absence of detergent. Finally F1, F2 and F3 were dialysed for 48 hours against 2 changes of 0.01M Tris HCl pH 8.0 buffer. The resulting solutions were concentrated with an ultrafilter and assayed for protein, lipid and cytochrome (see paragraph 6.4). These results summarised in Table 6.1 show that F1 and F2 are respectively protein-rich and lipid-rich fractions. Thus solubilisation of the membrane with MAD, rather than SDS permits the separation of most of the membrane lipid and protein by gel-filtration.

Fraction	Recovery of membrane constituents (percent)					
	Protein		Total lipid		Cytochrome	
	Strain 1	1M	1	1M	1	1M
F1	80	82	18	12	90	93
F2	16	14	82	87	7	5
F3	4	5	0	0	0	0

Table 6.1 Distribution of protein, lipid and cytochrome in fractions obtained by solubilising purified membrane with MAD followed by gel-filtration on Sephadex G100. Values are percentage of purified membrane content.

An amino acid analysis of F1 is shown in Table 6.2. The ratio of apolar to polar amino acids (H/A, see Chapter IV) is 1.4. This value is typical for membrane protein indicating an essentially hydrophobic protein.

lys	2.6	gly	9.3
his	1.4	ala	11.1
arg	3.0	val	7.2
asp	10.1	met	1.8
thr	6.3	ileu	4.9
ser	6.7	leu	9.3
glu	8.4	tyr	2.7
pro	5.2	phe	4.8

Table 6.2 Amino acid composition of the protein-rich fraction F1 obtained by treatment of strain 1 membrane with MAD. Results are given as mole%.

The protein-rich fraction, F1, could be sedimented when centrifuged at 150,000 x g for 2½ hours whilst the lipid-rich fraction F2 remained in the supernatant. A sample of F1 from strain 1 was centrifuged at 60,000 x g for 2½ hours on a 0-40% linear sucrose gradient. The distribution of material in the fractions collected when the centrifuge tube was emptied is shown in Figure 6.2 (a). A single cytochrome containing peak was detected in a position corresponding to the light fraction

from purified membrane. Thus F1 is not as dense as untreated purified membrane but penetrates the gradient considerably further than detergent disaggregated membrane.

An electronmicrograph of F1 prepared by the method described in paragraph 4.11 is shown in Figure 6.6. The stained sectioned material of F1 is fairly homogeneous, showing a lack of the prominent unit membrane structures which were evident in e.m.'s of purified membrane (see Figure 4.4). The occasional very small fragments showing a unit membrane like structure may represent a portion of membrane not fully solubilised by detergent.

Polyacrylamide gel electrophoresis: In Chapter IV it has been shown that purified membrane separates into only 3 bands when electrophoresed on polyacrylamide gels, most of the material including all of the carotenoid remaining at the origin. However, since detergents disaggregate the membrane into comparatively low M.W. particles a better separation of membrane components on P.G.E. might be expected in the presence of detergent. Figure 6.7 shows diagrams of gels obtained with membrane solubilised with MAD or SDS, and the fractions F1, F2 and F3 recovered from MAD treated membrane by gel-filtration.

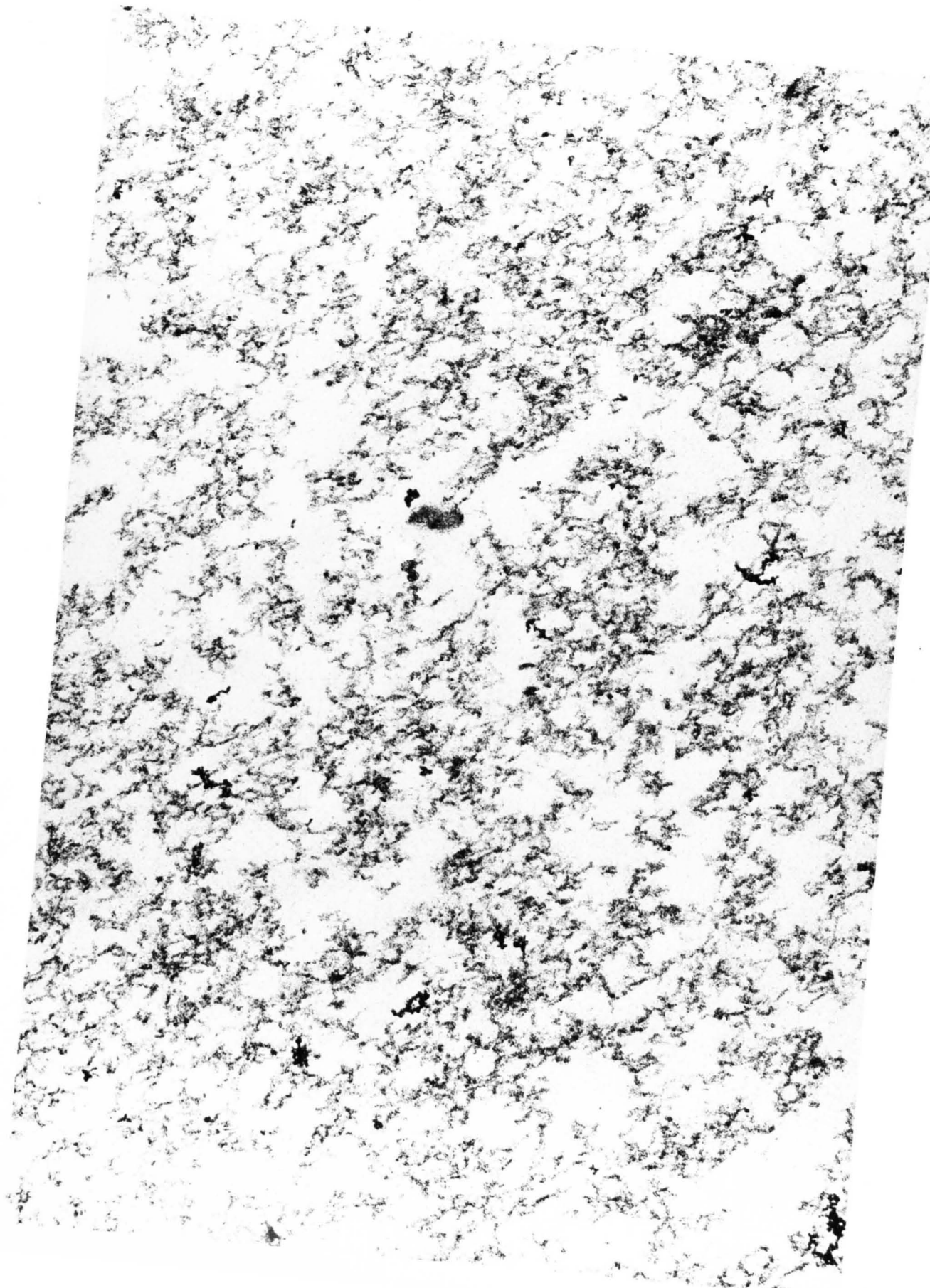
When SDS is present in the gel and electrode buffer almost all of the material moves at the migration front as a single red band. However, if detergent-free gels are used, an essentially similar

Figure 6,6

Electronmicrograph of F1, the protein-rich fraction obtained by gel-filtration on G100 of MAD solubilised strain 1 membrane (magnification x 70,000).

Details of staining are given in paragraph 4.10.







### Figure 6.7

Polyacrylamide gel electrophoresis of strain 1  
purified membrane disaggregated with detergents

Details of the experimental procedure used are given in  
paragraphs 4.5 and 6.3. The gels were stained for protein or  
lipid.

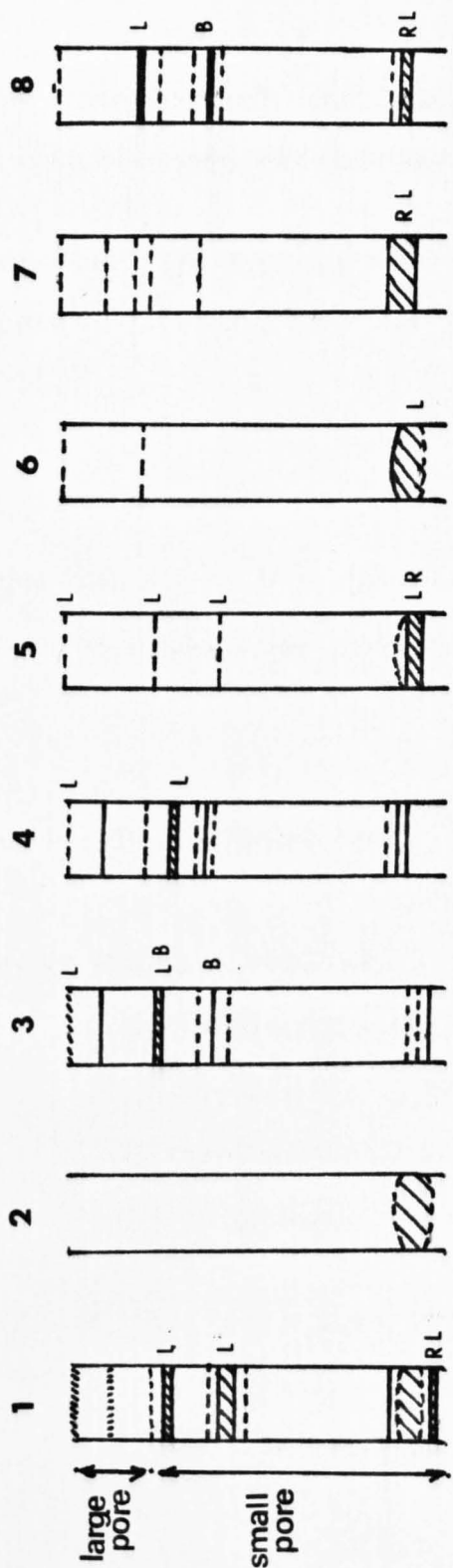
#### Key

1. Membrane + MAD (Membrane solubilised + MAD) was centrifuged  
at 150,000 x g for 2 hours and a sample of supernatant used  
for P.G.E.).
2. MAD alone.
3. Fraction F1 (no additional detergent, or when sample and gel  
contained 0.05% SDS).
4. Fraction F1 + 6M urea (Gel also contained 6M urea).
5. Fraction F2 (no additional detergent).
6. Fraction F3 (no additional detergent).
7. Membrane + SDS (Gels contained 0.05% SDS).
8. Membrane + SDS (No SDS in gels).

L = band stained for lipid or lipoprotein

R = band coloured red

B = band coloured brown



pattern of bands is obtained with MAD or SDS solubilised membrane.

The most prominent features of the gels are 2 slow moving lipoprotein bands and a fast moving lipid band which in strain 1 material is coloured red. This band is not present in F1 but is the pre-dominating component of F2. In addition F2, and F3 to a greater extent, contain a band near the front corresponding to detergent. Two weakly staining protein bands are present on the F2 gels, but they are also present in F1 which has the same distribution of bands as a total MAD-lysate sample with the exception of the red lipid front band. The slow-moving lipoprotein bands are brownish in colour before staining irrespective of whether the membrane is from strain 1 or 1M and may represent cytochrome containing material. F1 dissolved in 8M urea and run on gels containing 6M urea gave an essentially similar distribution of bands as urea-free gels. One component, the slowest moving prominent band, appears to have moved through the gel to a slightly greater extent in the presence of urea.

Analysis of lipids and cytochromes in F1 and F2: Total

lipids were extracted from F1 and F2 by the  $\text{CHCl}_3:\text{CH}_3\text{OH}$  procedure and analysed by T.L.C. as previously described (see paragraphs 5.1 and 5.2). The results obtained using a  $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}$  system to separate polar lipids are shown in Figure 6.8. Comparable quantities of each sample were applied i.e. 50-100  $\mu\text{g}$ . The lipid deficient fractions F1, from strain 1 and 1M, do not contain complete sets of components. Spot 3, which is an important

### Figure 6.8

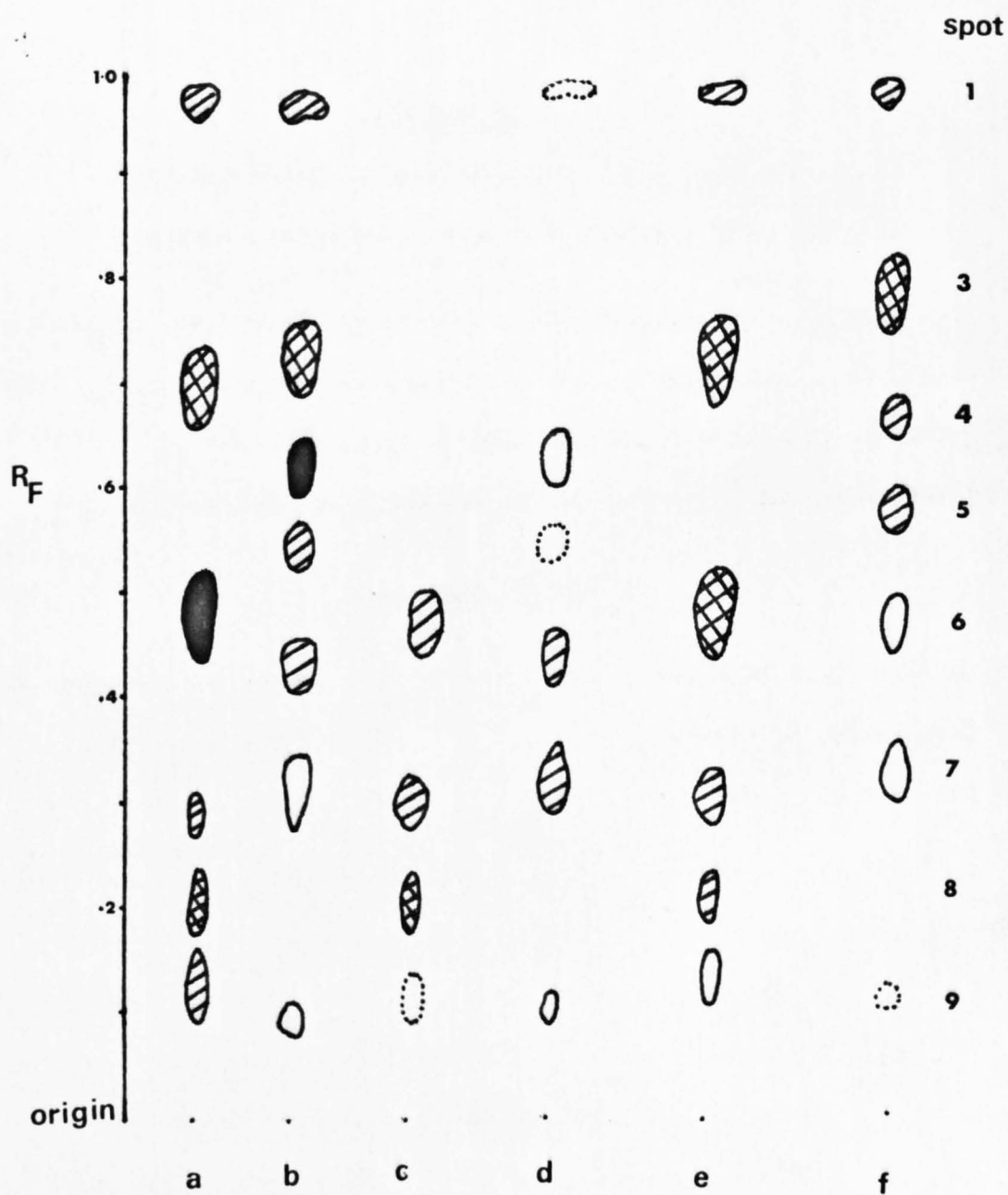
T.L.C. of total lipid extracts from purified membrane,  
and fractions obtained from MAD solubilised membrane

Plates were developed in a solvent containing  $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}$   
(65:25:4). Spots were visualised by charring with 40%  $\text{H}_2\text{SO}_4$ . The  
intensity of the spots is indicated by shading:

Black > crossed-line > diagonal line > open line > dotted line

### Key

- |                           |                            |
|---------------------------|----------------------------|
| (a) strain 1 membrane     | (d) strain 1M, fraction F1 |
| (b) strain 1M membrane    | (e) strain 1, fraction F2  |
| (c) strain 1, fraction F1 | (f) strain 1M, fraction F2 |



constituent of the total membrane extracts is missing from F1 of both strains. Also, as indicated by shading in Figure 6.8, the relative amounts of some of the remaining constituents is different from the whole membrane extract. Spot 6, the most prominent component of the strain 1 membrane polar lipids is slightly less prominent than spot 8 in the F1 extract. Similarly, with the mutant, the F1 extract contains more of the polar lipids and rather less of the less-polar components which were prominent in total membrane extracts e.g. Spots 4 and 5.

The F2 fractions which contain 80-90% of the extractable membrane lipid, in strain 1 and 1M show all of the polar lipids typical of whole membranes. However, the less-polar constituents are more prominent than in the F1 extracts and Spot 3 which was absent from F1 is present in F2 extracts.

The distribution of cytochromes in F1 and F2 from mutant membrane was compared with purified untreated mutant membrane. The absence of carotenoid in the mutant facilitated the interpretation of oxidised versus reduced difference spectra. Masking of peaks by the pigment had been suspected in strain 1 membrane preparations (Lancashire, 1970) and this effect would be exaggerated in F1 and F2 from strain 1 since the fractionation involves the separation of carotenoid into the lipid-rich fraction. Lancashire (1970) has reported finding no difference in the

constituent cytochromes of strain 1 and 1M.

Measurements were carried out using  $\text{Na}_2\text{S}_2\text{O}_3$  as reducing agent on a Bristol split-beam spectrophotometer operated by Mr W E Lancashire. Table 6.3 summarises the results obtained. A B type cytochrome was detected in F2 but the A and C type cytochromes were not found. The presence of some of the cytochromes in F1 was not confirmed, possibly due to the low concentrations of material available.

Type of Cytochrome	Wavelength of $\alpha$ peak (nm)		
	Purified Membrane	F1	F2
B type	558	(558)	558
	561	561	—
C type	553	554	—
	594	(594)	—
A type	610	(610)	—

Table 6.3 Classes of cytochrome present in purified strain 1M membrane and MAD solubilised membrane fractions. (—) indicates peak not confirmed by reproducible spectra.



## Discussion

The addition of detergents to suspensions of the purified membrane caused a marked clarification of the solution. The anionic detergent, SDS, was more effective than the non-ionic detergent Triton X-100. A similar result was reported by Razin and Barash (1969) when they attempted to solubilise the cell membrane of Mycoplasma laidlawii.

The particles of membrane material resulting from dissolution with the anionic detergents SDS or MAD (mixed anionic detergents) were of relatively low molecular weight since they were not sedimented by centrifuging at 150,000 x g for several hours on sucrose gradients. When SDS solubilised strain 1 membrane was eluted with buffer containing SDS from a Sephadex G100 column, protein and carotenoid were recovered in a single peak indicating a M.W. of 130,000. Since carotenoid can be used as a marker for membrane lipid this result implies that the membrane breaks down into lipoprotein particles. Also when SDS solubilised membrane was examined by P.G.E. with SDS in the gels almost all of the material migrated rapidly at the front as a single band. However, if SDS was absent from the G100 or acrylamide gels rather different results were obtained. Upon gel-filtration all of the membrane protein and carotenoid was recovered in a single peak in the column void volume indicating a M.W.  $>$  150,000. On P.G.E., however, protein and lipid components

appeared to be separated to some extent into a distinct red band migrating rapidly and several slower moving protein or lipoprotein bands. A similar result was obtained with MAD solubilised membrane on P.G.E. This implies that the low M.W. particles released on solubilisation with detergent do not necessarily represent lipoprotein sub-units of the membrane but merely aggregates of lipid-protein-detergent which can be fractionated by the high resolving capacity of P.G.E.

An interesting result was obtained with MAD solubilised membrane when this was subjected to gel-filtration on detergent free G100. The membrane material was recovered in 2 distinct fractions, F1 a protein-rich fraction of high M.W. and F2 a lipid-rich fraction of somewhat lower M.W.

F1 sedimented on a sucrose gradient in a similar position to purified membrane, but electron microscopy did not reveal prominent vesicles or portions of unit membrane as did the purified membrane preparations. However, structures bearing a close similarity to unit membrane were visible. These may represent undissociated portions of the original membrane sample or reaggregates which have formed upon removal of MAD by gel-filtration. Lipid extraction of F2 showed that this fraction contained almost 80-90% of the membrane lipid. Thus it is possible that the remaining 10-20% lipid and membrane protein can interact in such a way as to show the unit membrane type of structure. Terry (1966) showed that 95% of the

lipid of Mycoplasma laidlawii could be extracted with preservation of the triple-layered unit membrane structure.

Extraction of the lipids of F1 revealed upon analysis with T.L.C. that they were predominant polar phospholipids. The fact that the lipid was readily extractable with  $\text{CHCl}_3:\text{CH}_3\text{OH}$  suggests that hydrophobic bonds were involved between the protein and lipid. A relatively high proportion of apolar amino acids in F1 was demonstrated by amino acid analysis and this would tend to favour apolar protein-lipid interactions. Evidence provided by Reynolds and Tanford (1970) suggests that amphiphilic substances like anionic detergents and polar phospholipids react with membrane protein via hydrophobic bonds. Solubilisation of membrane proteins and lipids with detergents is an accepted technique for distinguishing hydrophobic bands from ionic, covalent or hydrogen bonding (Wallach, 1969). However, treatment of F1 with SDS or urea did not cause any further significant disaggregation when examined by P.G.E. Thus the possibility remains that covalent or ionic linkages are involved. However, F1 does not seem to be markedly affected by the presence of salts, although a slightly lower recovery of proteins was obtained in the pellet upon centrifugation at  $150,000 \times g$  of F1 which had been dialysed against  $4M$  NaCl. Presumably at this concentration of salt any ionic linkages in F1 would be disrupted.

Thus it appears that a proportion of the membrane lipid (up

to about 20%) may be bound to the protein in a different manner or rather more tightly than the remainder. This observation has been noticed by other workers using different membrane systems, Lenard and Singer (1968), Gordon et al (1969), Finean and Martenosi (1965) and Glaser et al (1970). Glaser et al found that about 75% of polar lipid of erythrocyte membrane was affected by temperature increases or attack by phospholipase. The remaining 25% was not accounted for. Of course, it is possible that removal or modification of the bulk of the membrane lipid caused a modification of the protein conformation which results in the remaining lipid becoming inaccessible to further enzyme attack or protected and stabilised to temperature changes. However, Glaser et al showed that a large fraction of the erythrocyte membrane lipid could be radically altered by heat or enzyme treatment without any detectable effect on the conformation of membrane protein.

It is possible that F1 represents partial reaggregation of protein and lipid upon removal of MAD by gel-filtration on G100. The eluting buffer contains 100mM NaCl which may aid reaggregation. Rottem et al (1968a) found that low concentrations of NaCl, about 250mM, caused a small degree of reaggregation of Mycoplasma membrane which had been disaggregated with detergent. However, complete reaggregation of F1 and F2 may require excess cations. In the next chapter experiments are described in which reconstituted membrane was prepared from F1 and F2 and the effects of various disaggregating agents investigated.

## Chapter VII : The reaggregation of detergent solubilised membrane

### Introduction

The interactions of membrane proteins and lipids have frequently been studied by investigations involving the reassembly of membrane dissociated with detergents or organic solvents (Rodwell et al, 1967; Butler et al, 1967; Rottem et al, 1968b; Razin and Bochwitz, 1968; Lenaz et al, 1970). By studying the conditions under which reaggregation occurs these investigations have attempted to obtain information which would give an indication of the nature of the bonding between the interacting components of the membrane. In this way, it has been shown that a low ionic strength, particularly including divalent cations, favours the formation of reaggregated membrane. Reconstituted membranes tend to be remarkably similar to native membrane preparations especially in their sensitivity towards disaggregating agents and behaviour on density gradients.

In this chapter experiments are described in which the reaggregation of the protein-rich fraction F1 and lipid-rich fraction F2 derived from detergent solubilised strain 1 membrane was carried out. The fractions were purified by dialysis or gel-filtration and mixed under a range of conditions including variation of pH, ionic strength and temperature.

Reconstituted membrane was isolated and compared with native

membrane by characterisation of its properties using electron microscopy, centrifugation, P.G.E., gel-filtration and effect of disaggregating agents. Experiments were also performed in which the fluorescent probe, 5-dimethyl-amino-1-naphthalene sulphonylchloride (DNS) was conjugated to native membrane, reconstituted membrane, and the fractions F1 and F2. Previously, important information on the conformation and on the motion of proteins has been obtained by the use of fluorescent probes covalently bound to amino acid side chains. (Weber, 1952; Weber and Young, 1964). By measuring fluorescence polarisation (p) changes in the internal freedom of rotation of polypeptide chains to which dansyl groups are bound can be detected, whilst fluorescent intensity reflects changes in the immediate molecular environment of the dansyl groups and can be used to detect conformational changes (Horton and Koshland, 1967). Using these techniques it was hoped to investigate the effect of applying various constraints e.g. heat, increasing ionic strength, to native membrane, reconstituted membrane and fractions F1 and F2.

## Section I : Methods

**7.1 Characterisation of reconstituted membrane:** Native membrane was solubilised with MAD and fractionated as described in Chapter VI. Reconstituted membrane was characterised by gel-filtration, sucrose density gradient centrifugation, P.G.E. and electron microscopy using the procedures described in Chapters IV and VI.

**7.2 Preparation of Dansyl conjugated membrane samples:** Reaction of a protein with 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride, DNS) can be achieved by exposing the protein to suitable concentrations of DNS under slightly alkaline conditions, (Horton and Koshland, 1967).

Dansylation was carried out by adding 0.1 or 0.2 ml of 5mM DNS (Sigma) in acetone to 1.0 or 2.0 ml of protein (including membrane samples) solution containing 5.0 or 10.0 mg of protein per ml in 5mM Tris HCl pH 8.0. The mixture was shaken and left at 4°C overnight. Unreacted DNS was removed by gel-filtration through a 2.0 x 55 cm column of G25 equilibrated with 5mM Tris HCl pH 8.0, (Gray and Hartley, 1963). The fractions collected in the column void volume contained dansylated material. These fractions were combined and dialysed for 24 hours against 5mM Tris HCl pH 8.0 at 4°C to remove last traces of "free" DNS.

The degree of dansylation was determined spectrophotometrically at 340 nm ( $A = 4.3 \times 10^3$ , Kasai et al, 1970). Absorbance values

at 340nm were corrected by control readings with undansylated samples of the same protein concentration. Mutant membrane was used to diminish quenching effects in the region of DNS fluorescence emission, i.e. 500-520nm.

7.3 Pronase digestion of dansylated membrane: Dansylated membrane was digested with Pronase at 37°C for 36 hours in a medium containing 0.5 mg of membrane protein, 0.1 mg of Pronase (Calbiochem) and 20mM sodium phosphate pH 7.0 in a total volume of 1.0 ml.

7.4 Measurement of fluorescence: Fluorescence intensity ( $I_f$ ) measurements were carried out using a Zeiss PMQII spectrophotometer fitted with fluorometric accessories. Fluorescence emission was measured at room temperature with exciting light of wavelength 365nm. The instrument was standardised using buffer and a quinine sulphate solution (1.0 mg/l in 0.1N  $H_2SO_4$ ) to give 0% and 100% transmitted light at 460 nm respectively.

Polarisation of fluorescence (p) was calculated from measurements of  $I_f$  made with a Farrand Mark I spectrofluorimeter. Measurements were made at room temperature and with slit widths of 20mμ. The instrument was standardised using a perspex standard excited at 360nm with emission measured at 420nm.  $I_f$  was measured for each sample with four different settings of the polariser and analyser prisms. Thus (p) may be defined as (Swoboda, 1969; Weill and Calvin, 1963):-



$$p = \frac{V_v - KH_v}{V_v + KH_v}$$

H and V refer respectively to  $0^\circ$  and  $90^\circ$  positions of analyser

h and v refer respectively to  $0^\circ$  and  $90^\circ$  positions of polariser

$K = \frac{V_h}{H_h}$  and is a correction factor for non-ideal transmission of polarised light through the monochromators.

## Section II : Results

### 7.5 Reaggregation of MAD solubilised membranes

SDS - solubilised strain 1 membrane reaggregates after extensive dialysis in the presence of 10mM  $MgCl_2$  to give a structure similar in appearance and behaviour on gel-filtration and sucrose gradients to the original purified membrane (Brown, Bellingham and Stevenson, 1971).

A series of experiments were undertaken to investigate further the nature of reconstituted membrane. Firstly the effects on the process of reaggregation by a range of salts, pH and temperature were studied.

#### The effect of salts on the reaggregation of F1 and F2:

Fractions F1 and F2 were prepared from strain 1 membrane as described in paragraph 6.6. The total amounts of each purified fraction obtained from a given sample of MAD solubilised membrane were mixed and dialysed at 4°C for 12 hours against several changes of 0.01M Tris HCl pH 8.0 containing the various salts under investigation. After 12 hours the contents of the dialysis bags were centrifuged at 150,000 x g for 2½ hours. The resulting pellets were washed with 0.01M Tris HCl pH 8.0, recentrifuged and the washings combined with the supernatants. These final supernatants and pellets, resuspended in Tris HCl buffer were assayed for protein and carotenoid. Results are summarised in

Table 7.1. Levels of protein and carotenoid are expressed as percentages of the original untreated membrane, i.e. their recovery in the pellet was used as an estimate of reaggregation. Protein was determined by the Folin method and carotenoid by the procedure described in paragraph 4.8. Reaggregation of F1 and F2 into material sedimentable by centrifugation at  $150,000 \times g$  and containing a similar distribution of protein and carotenoid as untreated membrane only occurred after dialysis of F1 and F2 in the presence of  $Mg^{2+}$  or the polycation spermine. The pellets obtained in the presence of NaCl KCl and  $CaCl_2$  represent F1 which is sedimented under these conditions.

Electronmicrographs of material reconstituted in the presence of  $10mM Mg^{2+}$  and  $5mM$  spermine are shown in Figure 7.1.

The effect of pH and temperature on the reaggregation of F1 and F2: Purified preparations of strain 1 F1 and F2 were mixed and dialysed for 12 hours at  $4^{\circ}C$  against buffers of pH 5.0, 7.0 and 9.0 containing  $10mM MgCl_2$ . The dialysates were centrifuged at  $150,000 \times g$  for  $2\frac{1}{2}$  hours and the resulting washed pellets and supernatants assayed for protein and carotenoid.

In other experiments F1 and F2 mixtures were dialysed at  $4^{\circ}$ ,  $25^{\circ}$  and  $40^{\circ}$  against  $0.01M$  Tris HCl pH 8.0 containing  $10mM MgCl_2$ . Aliquots of one third of the dialysate were taken at time intervals of 0, 6 and 12 hours, centrifuged and assayed as described above. Results are summarised in Tables 7.2 and 7.3.

Reconstituting Medium	Recovery of protein and carotenoid (%)			
	Pellet		Supernatant	
	Protein	Carotenoid	Protein	Carotenoid
Control	100	100	100	100
Distilled H <sub>2</sub> O	84.0	2.0	15.0	94.0
MgCl <sub>2</sub> 10mM	99.0	100	1.0	0
MgCl <sub>2</sub> 100mM	97.0	100	1.5	0
NaCl 100mM	80.0	0	18.0	96.5
KCl 100mM	78.0	0	16.5	98.0
CaCl <sub>2</sub> 100mM	81.5	1.2	15.0	93.0
Spermine 5mM	99.0	100	0	0

Table 7.1 The effect of salts on the reaggregation of MAD solubilised strain 1 membrane. Mixtures of fractions F1 and F2 were dialysed against 0.01M Tris HCl pH 8.0 containing various salts. After dialysis the contents of the bag were centrifuged at 150,000 x g for 2½ hours. (see paragraph 6.7). The control contained untreated membrane in Tris HCl buffer only.

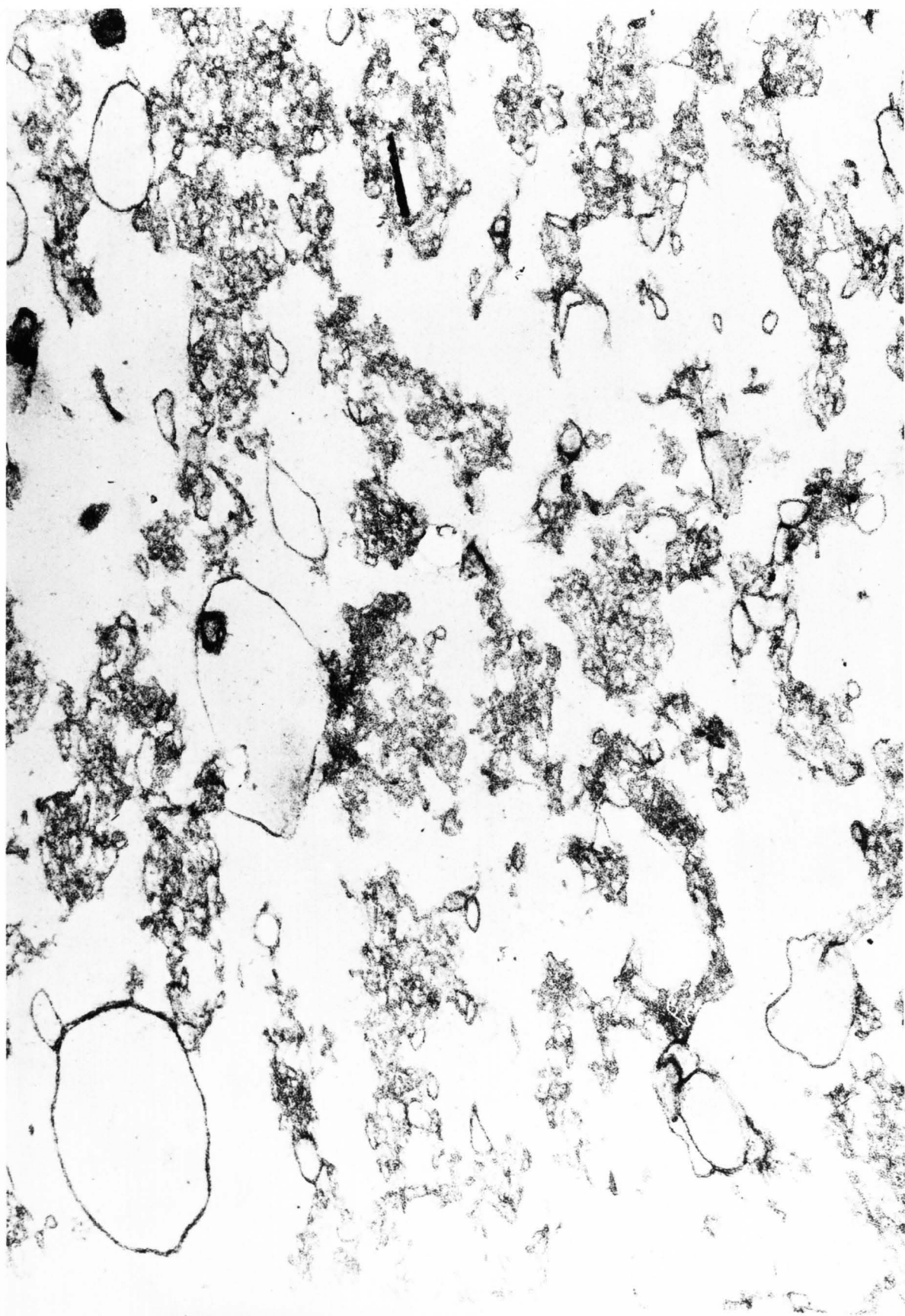
Figure 7.1

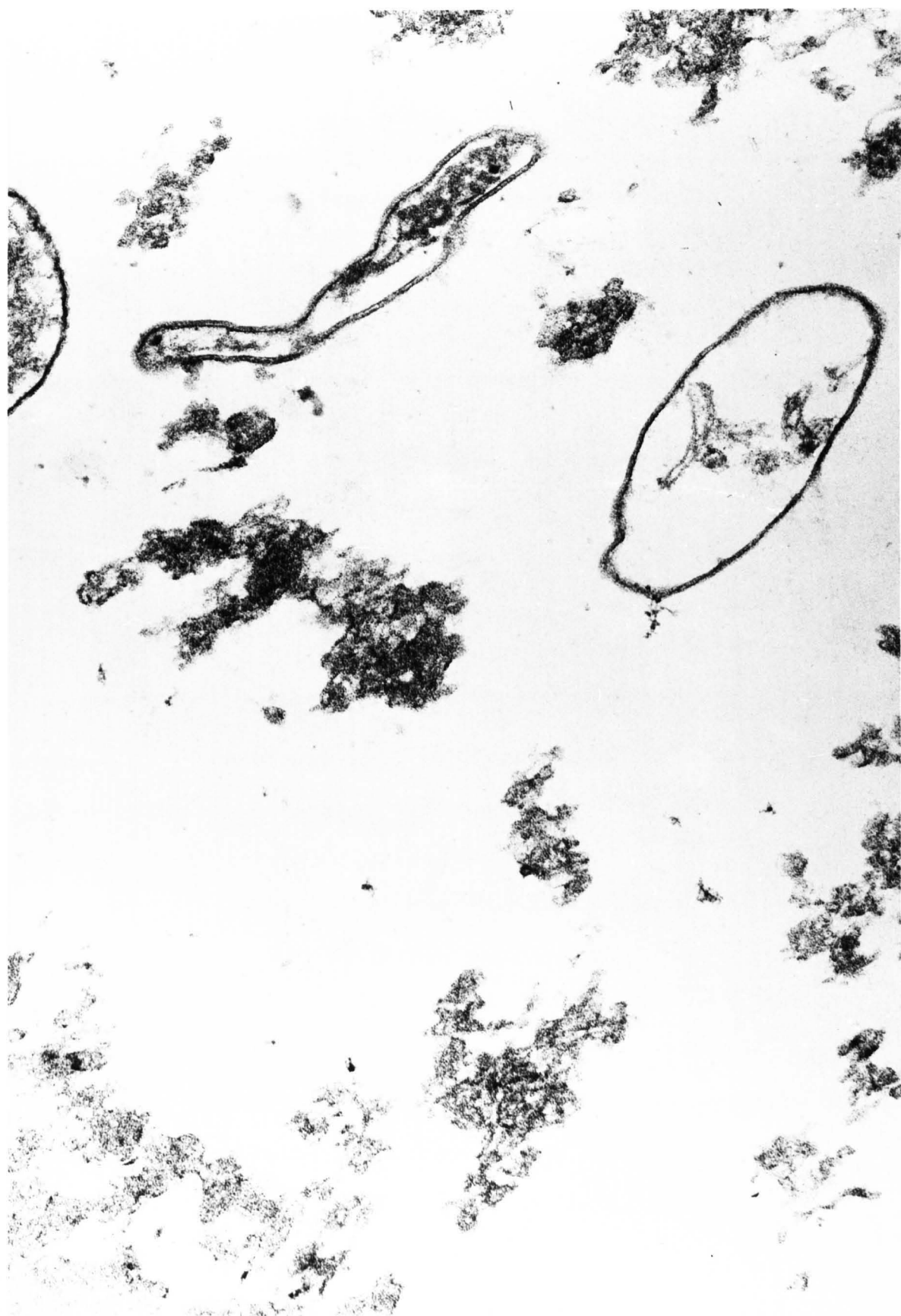
Electronmicrographs of strain 1 membrane reconstituted from MAD  
solubilised membrane (magnification x 70,000)

Mixtures of F1 and F2 were dialysed for 12 hours at 4°C  
against 0.01M Tris HCl pH 8.0 containing either 10mM  $Mg^{2+}$  or  
5mM spermine. The contents of the bags were centrifuged at  
150,000 x g for 2½ hours, washed, respun and the final pellets  
prepared for electronmicroscopy as described in paragraph 4.11.

Plate I : Reconstituting medium contained 10mM  $MgCl_2$

Plate II : Reconstituting medium contained 5mM spermine





pH of Buffer		Recovery of Protein and Carotenoid (%)			
		Pellet		Supernatant	
		Protein	Carotenoid	Protein	Carotenoid
5.0	Control	94	100	1	0
	Test	80	100	12	0
7.0	Control	100	100	0	0
	Test	95	100	2	0
9.0	Control	98	100	0	0
	Test	100	98	0	0

Table 7.2 The effect of pH on the reaggregation of MAD solubilised strain 1 membrane. Mixtures of F1 and F2 were dialysed against the following buffers containing 10mM  $MgCl_2$ :

pH 5.0 : 0.05M Sodium citrate

pH 7.0 : 0.05M Tris maleate

pH 9.0 : 0.05M Tris HCl

The dialysates were centrifuged at 150,000 x g for 2½ hours, washed and respun. Protein and carotenoid of the pellets and supernatants was determined. Controls were unsolubilised purified membranes dialysed for 12 hours against the reconstituting buffers.



		Recovery of Protein and Carotenoid (%)											
		0 hours				6 hours				12 hours			
		Pellet		Supernatant		Pellet		Supernatant		Pellet		Supernatant	
		P	C	P	C	P	C	P	C	P	C	P	C
4°C	Control	100	100	0	0	100	100	0	0	100	100	0	0
	Test	75	0	16	100	78	0	10	97	98	97	1	0
25°C	Control	100	100	0	0	100	100	0	0	100	100	0	0
	Test	80	0	15	97	98	82	4.5	8.6	98	100	3.4	0
40°C	Control	100	100	0	0	97	100	0	0	100	100	0	0
	Test	77	0	15	100	93	71	14	20	100	95	0	0

Table 7.3 The effect of temperature on the rate of reaggregation of MAD solubilised strain 1 membrane. Mixtures of F1 and F2 were dialysed against 0.01M Tris HCl pH 8.0 at various temperatures. Controls at each temperature were samples of untreated membrane. Periodically aliquots were removed, centrifuged and the pellets and supernatants assayed for protein and carotenoid.

P = protein      C = carotenoid

## 7.6 The effect of disaggregating agents on reconstituted membrane

The susceptibility of reaggregated membrane to treatment with disruptive agents was tested in an attempt to investigate the nature of the binding between the recombined membrane constituents. A range of reagents was chosen which have previously been used to disaggregate protein or lipoprotein.

For each test strain 1 reconstituted membrane was prepared by dialysis of F1 and F2 against 10mM  $Mg^{2+}$  and 0.01M Tris HCl pH 8.0 for 14 hours at 4°C. The resulting pellet was washed at least twice with the same buffer before treatment with the disaggregating agent under test. The extent of solubilisation of the membrane was determined by monitoring the level of protein and carotenoid in the recovered membrane or membrane fractions.

Increasing salt concentration: The effect of NaCl was tested by dialysing reconstituted membrane samples (about 10 mg protein) for 14 hours at 4°C against 0.01M Tris HCl pH 8.0 buffer containing zero, 2M or 4M NaCl. The contents of the bags were then centrifuged at 150,000 x g for 2½ hours. The recovery of protein and carotenoid in the washed pellets and supernatants was determined. Samples of crude and purified membrane, F1 and F2 were also included in the experiment since previously Larsen (private communication) had reported that freshly prepared membrane tended to precipitate in the presence of 25% NaCl. Results are shown in Table 7.4.

Sample	NaCl M	Recovery of protein and carotenoid (%)			
		Pellet		Supernatant	
		Protein	Carotenoid	Protein	Carotenoid
Freshly prepared crude membrane	0	100	100	0	0
	2	98	100	0	0
	4	reddish-pink precipitate			
Purified membrane	0	97	100	0	0
	2	98	100	1	0
	4	100	98	0	0
Reagg. membrane	0	98	100	0	0
	2	100	97	0	0
	4	97	96	0	0
F1	0	100	-	0	-
	4	94	-	0	-
F2	0	0	0	92	98
	4	2 layers } red upper } colourless } lower		- 10	100 0

Table 7.4 The effect of salt on strain 1 crude and purified membrane and membrane reconstituted from F1 and F2. See paragraph 6.8 for experimental details.

- indicates not tested

Urea: In an experiment carried out by R H Brown purified strain 1 membrane was suspended in 0.01M Tris HCl pH 8.0 containing 8M urea and subjected to gel-filtration on an Agarose A50m column equilibrated with the same buffer containing 6M urea, (Brown, Bellingham and Stevenson, 1971). This experiment was repeated using reconstituted membrane.

About 80% of the protein and all of the carotenoid and cytochrome of the sample was recovered in a single peak eluted in the column void volume. The rest of the protein was eluted as a rather broad peak immediately following the void volume with an  $\frac{E_v}{E_o} \approx 1.2$ . This corresponds to a particle M.W. of about 700,000 (see Figure 4.2). Brown, however, found that although all of the lipid and cytochrome were eluted in the void volume only about 60% of the membrane protein was present in this peak. The remainder was recovered from 2 peaks, 30% as a protein corresponding to a M.W. of 800,000 and approximately 5% in the column bed volume with a M.W. of about 2500. He also observed that the release of protein from the membrane caused by urea could be reduced to about 10% by the presence of 1mM  $Mg^{2+}$  in the eluting buffer.

As a further characterisation purified membrane and reconstituted membrane suspended in Tris HCl-6M urea were analysed by P.G.E. in which the gels were supplemented with 6M urea. Diagrams of the resulting gel are shown in Figure 7.2.

Ethylenediaminetetra acetic acid (E.D.T.A.): E.D.T.A. is a well known chelating agent which forms stable complexes with  $Mg^{2+}$ . To extract  $Mg^{2+}$  from membrane samples, purified membrane and reconstituted membrane preparations were dialysed at 4°C for 48 hours against at least 4 changes of 0.01M Tris HCl pH 8.0 containing 0.05% E.D.T.A. (di-sodium salt). The resulting solutions were applied to an Agarose A50m column and eluted with the same buffer. In both cases all of the protein, carotenoid and cytochrome was recovered in a single peak eluted in the column void volume.

Mixed anionic detergents: A solution of MAD solubilised reconstituted membrane was prepared by the same method as described in paragraph 6.6. The resulting solution was subjected to gel-filtration on Sephadex G100 and P.G.E. under similar conditions as described in paragraph 6.6. Reconstituted membrane labelled with  $^{32}P$  gave a similar distribution of protein and label in the G100 effluent as solubilised purified membrane (see Figure 6.4b). However, on P.G.E. a significant difference was apparent. A diagram of a typical gel is shown in Figure 7.2 gel 3. This shows carotenoid present in slow moving bands near the top of the small pore gel. By contrast, in the gels of  $Mg^{2+}$  free MAD solubilised membrane, and membrane solubilised in the presence of 10mM  $Mg^{2+}$  (Figure 6.7 and 7.2 respectively), the carotenoid is present in both cases as a prominent band near the bottom of the gels.

Figure 7.2

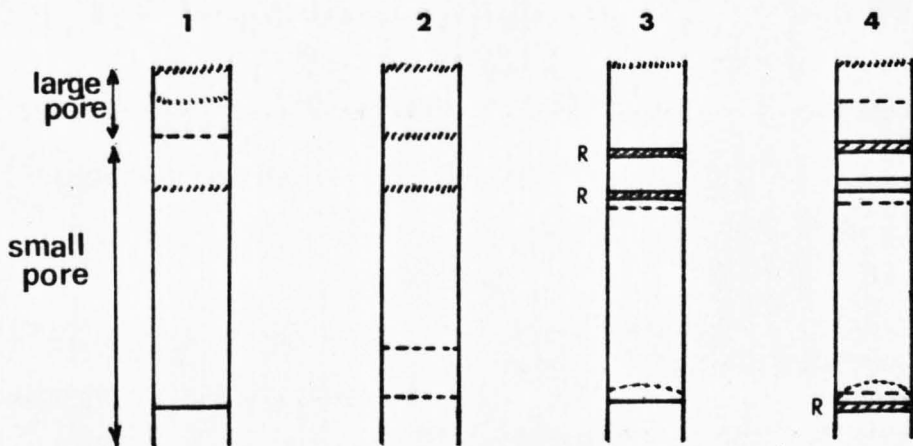
Polyacrylamide gel electrophoresis of purified  
and reconstituted strain 1 membrane

Details of the experimental procedures used are given in  
paragraphs 4.5 and 6.3. The gels were stained for protein.

Key

- |   |   |                                |
|---|---|--------------------------------|
| 1. Purified membrane + 6 <u>M</u> urea                                    | } | gels contained 6 <u>M</u> urea |
| 2. Reconstituted membrane + 6 <u>M</u> urea                               |   |                                |
| 3. Reconstituted membrane solubilised with MAD                            |   |                                |
| 4. Purified membrane + 10 <u>mM</u> $\text{Mg}^{2+}$ solubilised with MAD |   |                                |

R = red



### 7.7 Binding of DNS to purified membrane

DNS was conjugated to purified mutant membrane (M-DNS) and B.S.A. (BSA-DNS) by the method described in paragraph 7.1. The emission spectra obtained when labelled samples were excited at 365nm in the presence or absence of MAD solution are shown in Figure 7.3. The addition of MAD to M-DNS caused a 68% increase of fluorescence intensity ( $I_f$ ) accompanied by a 10nm shift of the emission maximum to the blue. The BSA-DNS, however, gave only a 16% increase of  $I_f$  on the addition of MAD and showed no shift of emission maximum.

In order to identify the membrane component(s) to which the DNS was binding aliquots of M-DNS were fractionated by lipid extraction on LH-20 or gel-filtration on G100 in the presence of MAD (see paragraphs 5.4 and 6.6). The fractions collected were assayed for the presence of dansylated groups by measuring their  $I_f$  at 510nm. Results are shown in Figure 7.4. In both cases DNS fluorescence appears mainly in the high molecular weight protein containing fractions (see Figures 5.8 and 6.4 for distribution of lipid in these fractionations). The protein-rich fraction from the G100 column (F1-DNS) was concentrated on an ultrafilter and its emission spectrum measured (see Figure 7.3b). On treatment with MAD,  $I_f$  increased by 55% with a slight change of emission maximum to a lower wavelength. F1-DNS was also treated with a

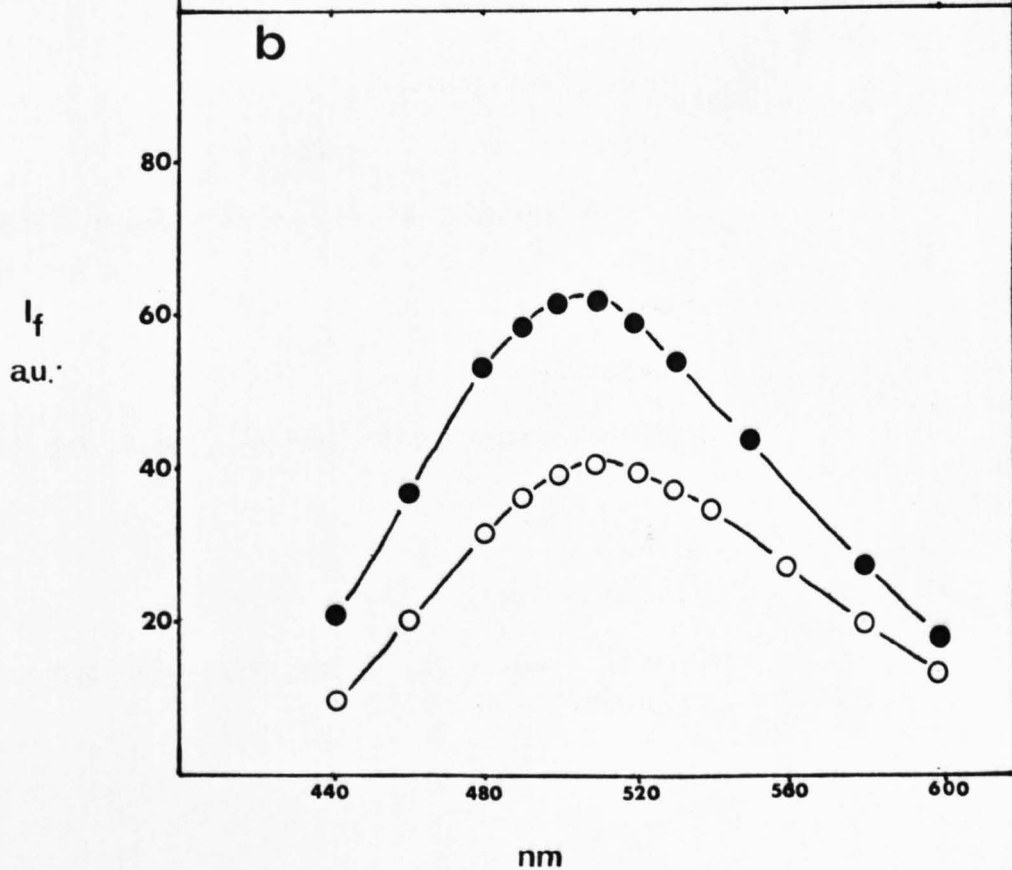
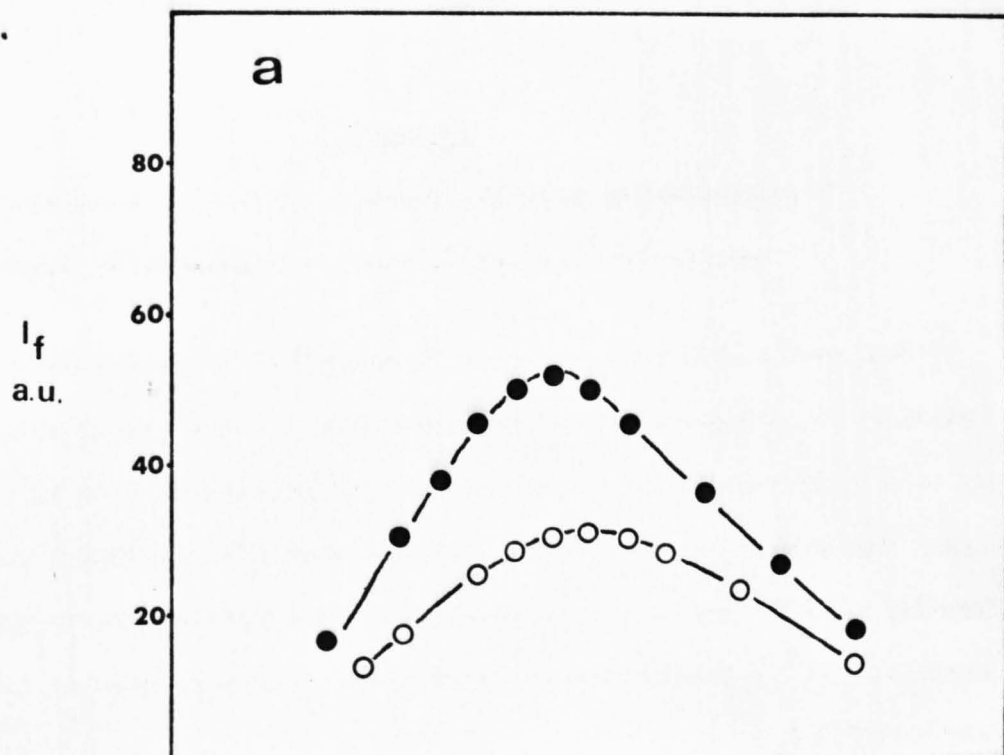


### Figure 7.3

Fluorescence emission spectra of strain 1M membrane-DNS  
conjugates and Bovine serum albumin-DNS conjugates

DNS conjugates were prepared as described in paragraph 7.1.  
Fraction F1, lipid deficient membrane, was prepared by solubilisation  
of strain 1M membrane with MAD by the method described in paragraph  
6.6. The samples of conjugates solubilised with detergent were  
treated with 0.2 ml MAD/10 mg protein. Fluorescence intensity  
measurements were made with a Zeiss PMQII spectrophotometer (see  
paragraph 7.3).

- (a) Purified membrane-DNS ○  
" " + MAD ●  
(0.25 mg protein per ml and 40 μ moles DNS per g)
- (b) F1-DNS ○  
F1-DNS + MAD ●  
(0.2 mg protein per ml and 45 μ moles DNS per g)
- (c) BSA-DNS ○  
BSA-DNS + MAD ●  
(0.2 mg protein per ml and 50 μ moles per g  
protein)



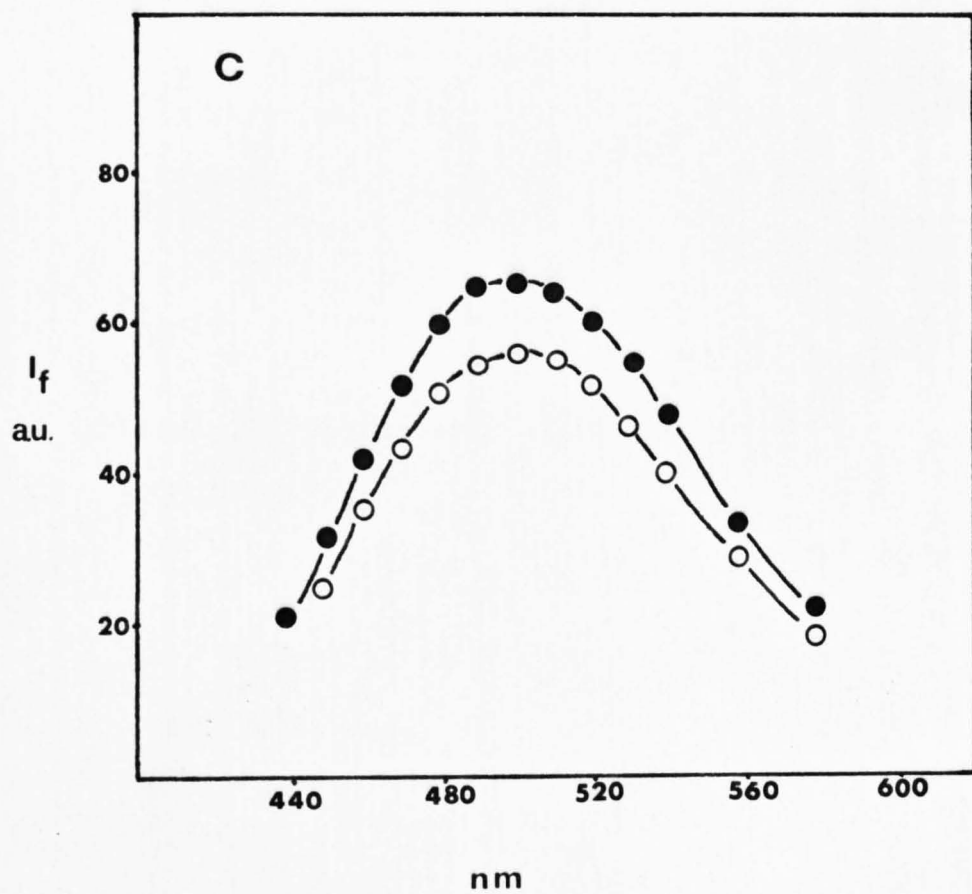


Figure 7.4

Characterisation of dansylated fractions  
of strain 1M membrane by gel-filtration

The  $I_f$  at 510nm of fractions eluted from Sephadex columns were determined with a Zeiss PMQII spectrophotometer (see paragraph 7.4).

Dansylated membrane was treated as follows:

- (a) Solubilised with MAD ( 0.2 ml MAD/10 mg protein) and eluted from a 1.5 x 50 cm Sephadex G100 column with 0.01M Tris HCl pH 8.0 containing 0.1M NaCl.
- (b) Solubilised with 90% 2-chloroethanol in 0.01N HCl and eluted with the same solvent from a 2.5 x 30 cm Sephadex LH-20 column.

----- Percentage transmission at 254nm

●  $I_f$  (a.u.)

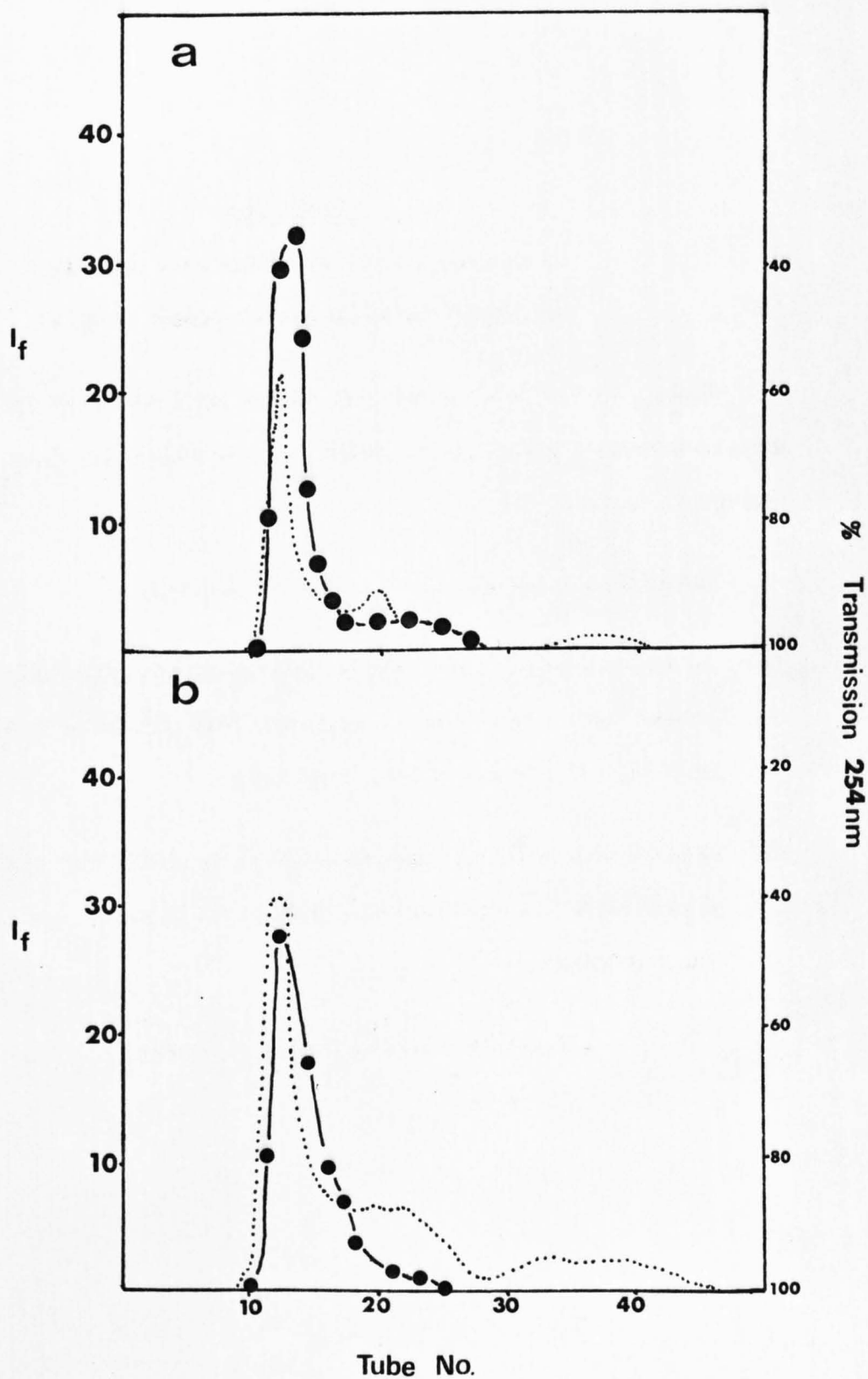


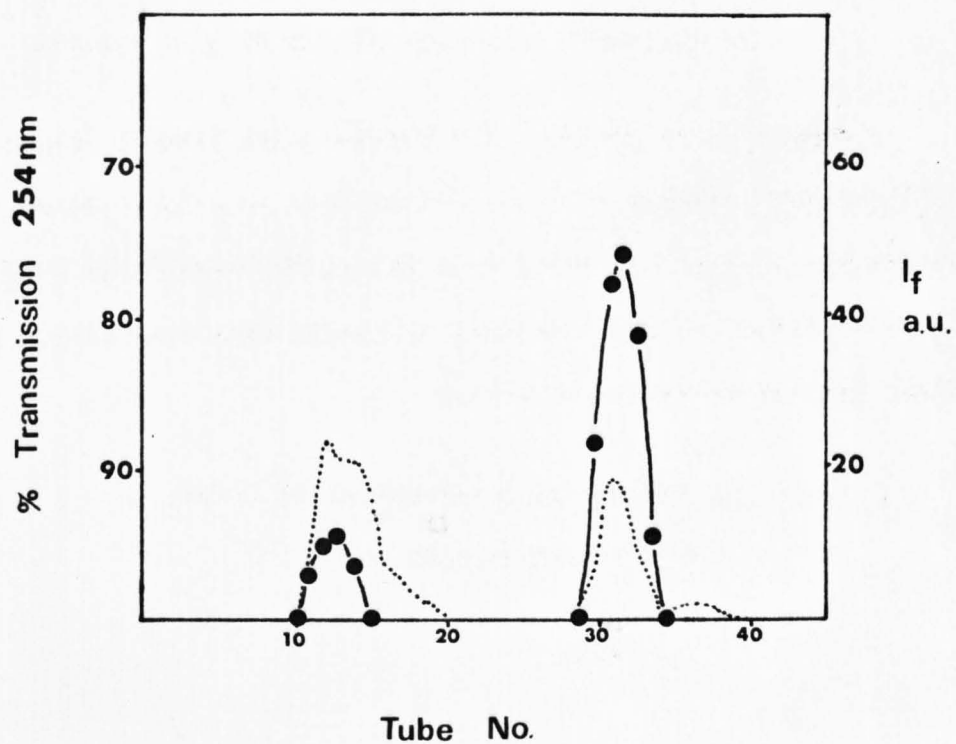
Figure 7.5

Gel-filtration on Sephadex G25 of membrane solubilised  
by digestion of strain 1M F1-DNS with Pronase

F1-DNS (0.5 mg protein) was treated with Pronase (see paragraph  
7.3) and the products of digestion applied to a 1.5 x 50 cm G25  
column and eluted with 0.01M Tris HCl pH 8.0 containing 0.1M NaCl.  
 $I_f$  measurements of the fractions collected were made with a Zeiss  
PMQII spectrophotometer at 510nm.

- - - - - Percentage transmission at 254nm  
of the column effluent

●  $I_f$  (a.u.)



prolonged digestion by Pronase, a protease of wide substrate specificity (Nomoto et al, 1960), (for conditions, see paragraph 7.3). The resulting solution contained 90% of the DNS, of which 80% was present in a low M.W. fraction (see Figure 7.5). The fraction probably contains free amino acids or short peptides liberated by Pronase and therefore implies that DNS is conjugated to proteins in F1.

#### 7.8 The effect of temperature on the polarisation of fluorescence of membrane bound DNS

Samples of labelled membrane containing 0.1-5.0 mg of protein per ml and up to 100  $\mu$  moles DNS per g of protein were equilibrated in a water-bath and maintained at temperature for 10 minutes before reading polarisation of fluorescence (p). Figure 7.6 shows the results obtained.

Reconstituted membrane was prepared by dialysing a mixture of F1 and F2, prepared from mutant M-DNS by MAD treatment, against 0.01M Tris HCl pH 8.0 containing 10mM  $MgCl_2$  for 15 hours at 4°C. Native membrane showed a marked decrease of p above 50°C, whilst reconstituted membrane decreased steadily. Samples of F1 and F2 prepared from unlabelled membrane and dansylated separately also showed a steady decrease of p values over the whole range of temperatures tested. The readings of F2-DNS, however, were very low and barely changed above 40°C.



Figure 7.6

The effect of temperature on the polarisation  
of fluorescence (p) of membrane bound DNS

Samples of dansylated material were maintained at temperature  
for 10 minutes and then (p) was measured at room temperature using  
a Farrand Mark I spectrofluorimeter (see paragraph 7.4).

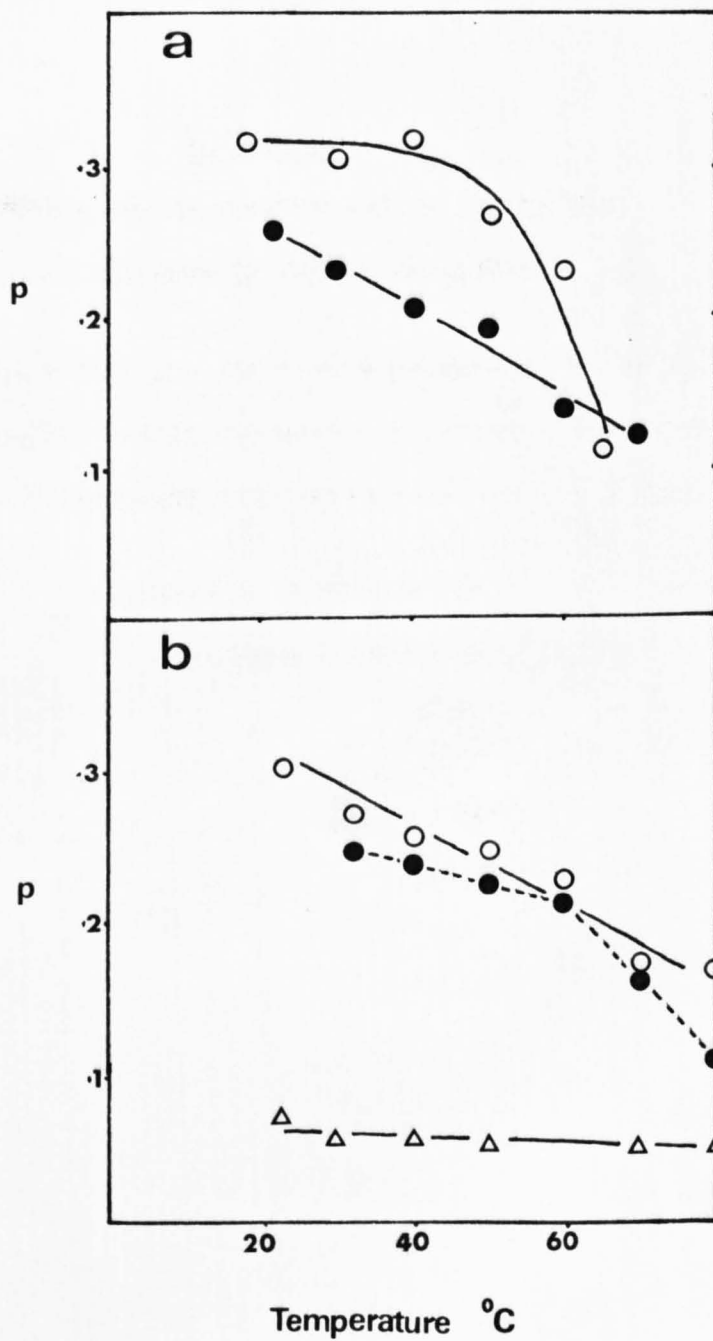
(a) Purified strain 1M membrane      ○

Reconstituted membrane      ●

(b) F1-DNS      ○

F2-DNS      Δ

BSA-DNS      ●



The result obtained with BSA-DNS tended to indicate a steady decrease similar to F1-DNS rather than the sudden change observed with native membrane.

#### 7.9 The effect of environmental viscosity on polarisation of fluorescence of bound DNS

Samples of DNS labelled membrane were treated with solid sucrose to give a final concentration of either 0.15M or 1.5M sucrose. The final solutions contained approximately 0.2 mg protein per ml. Readings of  $p$  were made at room temperature. Results are shown in Table 7.5.

Native membrane, reconstituted membrane and F1-DNS gave very similar values of  $p$  in the presence and absence of sucrose. F2-DNS, however, although with a much lower  $p$  value than the other samples showed an approximately 12.5% increase of  $p$  in the more viscous solution.

#### 7.10 The effect of salts on $p$ of membrane bound DNS

Polarisation of fluorescence is not influenced per se by factors such as charge or ionic strength (Horton and Koshland, 1967) and therefore changes in the conformation of protein-DNS conjugates induced by salts may be observed by fluorescence studies.

Preparations of M-DNS, (native membrane), RM-DNS (reconstituted membrane), F1-DNS (lipid deficient membrane fraction) and F2-DNS

	Polarisation of fluorescence (p)		
	0.15M Sucrose	1.5M Sucrose	Change of p
Native Membrane	0.310	0.315	0.005
Reconstituted membrane	0.295	0.299	0.004
F1-DNS	0.289	0.293	0.004
F2-DNS	0.081	0.094	0.013

Table 7.5 Variations of the polarisation of fluorescence of bound DNS with membrane environment. The viscosity of the medium, 0.01M Tris HCl pH 8.0, was increased by the addition of sucrose.

(lipid rich lipoprotein) were treated with NaCl or  $\text{MgCl}_2$ . Samples containing 1.0-5.0 mg protein per ml were dialysed at  $4^\circ\text{C}$  for 15 hours against at least 3 changes of buffer containing 5mM Tris HCl pH 8.0 and one of the salts within the range 1mM -1000mM. After dialysis bag contents were adjusted to 0.2-1.0 mg protein/ml by diluting with the same buffer. Controls were dialysed for the same time against Tris HCl buffer in the absence of NaCl or  $\text{MgCl}_2$ . In the experiment with  $\text{MgCl}_2$  a duplicate set of samples were dialysed for 24 hours against several changes of 0.05% EDTA followed by 24 hours against 5mM Tris HCl pH 8.0, before treatment with  $\text{MgCl}_2$  - Tris HCl buffer.

Fluorescence measurements were made at room temperature and results obtained are given in Table 7.6. Native membrane was insensitive to changes of NaCl and  $\text{MgCl}_2$ . Reconstituted membrane, however, showed a marked drop of p in 1M NaCl, although no significant changes were detected at the other levels of NaCl or  $\text{MgCl}_2$ . The membrane fractions F1-DNS and F2-DNS were sensitive to changes of  $\text{Mg}^{2+}$  and in addition F2-DNS showed a comparatively large drop of p in 1M NaCl. An increase of p in the presence of  $\text{MgCl}_2$  was only detected in F1-DNS samples predialysed against EDTA. F2-DNS on the other hand only showed a significant increase of p in the samples which had not been dialysed against EDTA.

	Polarisation of fluorescence (p)				
	Concentration of NaCl (mM)				
	0	1	10	100	1000
Native membrane	0.301	0.301	0.300	0.298	0.304
Reconstituted membrane	0.299	-	0.303	-	0.275
F1-DNS	0.300	0.303	0.299	0.286	0.301
F2-DNS	0.102	0.104	0.100	0.083	0.072

	Concentration of MgCl <sub>2</sub> (mM)						
	0 (a)	1 (a)	2	10 (a)	100		
Native membrane	0.312	0.307	0.304	0.308	0.310	0.307	0.301
Reconstituted membrane	0.302	0.301	-	-	0.303	-	-
F1-DNS	0.289	0.288	0.300	0.287	0.305	0.303	0.308
F2-DNS	0.096	0.087	0.091	0.094	0.100	0.101	0.109

Table 7.6 The effect of salts on the polarisation of fluorescence of dansylated membrane preparations.

(a) predialysed against 0.05% EDTA

## Discussion

Strain 1 purified membrane can be solubilised with MAD and fractionated to give a protein-rich fraction F1 and a lipid-rich fraction F2. Unit membrane structures are not conspicuous in electronmicrographs of F1, but under suitable conditions F1 and F2 will recombine to give structures which have a typical unit membrane appearance when seen on electronmicrographs. Reconstitution of membrane will only occur in the presence of  $Mg^{2+}$  and the polyvalent cation spermine.  $Ca^{2+}$  did not promote reaggregation and nor did the monovalent ions  $Na^+$  and  $K^+$  even at 100mM.  $Mg^{2+}$  and spermine on the other hand were effective at 10mM and 5mM respectively. In 10mM  $Mg^{2+}$  reaggregation occurred slightly more effectively at pH 7 and 9 than pH 5. At pH 8.0 and 10mM  $Mg^{2+}$  recombination was much faster at 25°C than 4°C being almost complete after 6 hours.

Thus it appears that the presence of certain cations is essential for reassembly to occur. Rottem et al (1968) working with Mycoplasma membranes found that the concentration of  $Mg^{2+}$  at low levels determined the ratio of protein to lipid in the reaggregates. For example, at 5mM  $Mg^{2+}$  the reaggregates contained much higher levels of lipid than native membrane. However, in the experiments with F1 and F2 reaggregation seems to be completed at 10mM  $Mg^{2+}$ . Reaggregation is temperature dependent, being much

faster at 25°C than 4°C. A similar result was obtained by Lenaz et al (1970) in studies on the binding of phospholipids to lipid-deficient mitochondria.

The limited electron microscope studies which were carried out show vesicles bounded by a triple-layered structure forming the predominant reaggregated membrane material. Similar structures have been reported in reaggregates of material from various membrane sources, e.g. Terry et al, 1967; Green et al, 1967 and Ernster et al, 1962. Large vesicles, with smaller vesicles inside are often seen. This is particularly evident in the ems shown in Figure 7.1. Some of the vesicles found in the presence of  $Mg^{2+}$  appear to branch forming 'Y' shaped junctions. A similar observation was reported by Chuang et al (1970) in reaggregates formed from mitochondrial phospholipids and cytochromeoxidase. In overall appearance the reassembled material is similar to native membrane although a substantial proportion is not clearly triple-layered and resembles the protein-rich fraction, F1 (Figure 6.6). A certain amount of this material i.e. not clearly triple-layered or vesicular is also present in some of the ems of native membrane especially crude strain 1 membrane (Figure 4.4). However, the reassembly of solubilised membrane lipid (F2) and protein (F1) upon removal of detergent suggests that these components retain sufficient structure determining information to interact spontaneously in the presence



of  $Mg^{2+}$  to produce membraneous structures essentially similar to native membrane in appearance. Although traces of triple layered structures are visible in the em of F1 it seems that large fragments and vesicles are only formed when the membrane protein reacts with the lipid fraction which contains predominantly neutral lipids.

The nature of the interaction between F1 and F2 was investigated by studying the effect of disaggregating agents on reconstituted membrane. The results indicate that the interaction is essentially hydrophobic in character. The general sensitivity of reconstituted membrane to disaggregation was similar to native membrane. Both are disrupted by MAD and the products, a protein-rich fraction and a lipid-rich fraction, can be separated by gel-filtration. However, on P.G.E. reconstituted membrane revealed an interesting difference to native membrane. One of the slow moving bands was missing from the large pore gel and carotenoid was present in slow moving material. This suggests that the carotenoid is rather more tightly bound in the reconstituted material, possibly inaccessible to attack by detergent since in native membrane carotenoid is always present in a fast moving band after treatment with detergent. Treatment of reconstituted membrane with urea followed by fractionation on Agarose also revealed that a protein present in native membrane was absent. The reassembled lipoprotein seemed to be less susceptible to disruption with urea since only

20% of the protein was eluted in the column bed volume whereas up to 35% was solubilised from native membrane. It is interesting to note that release of these fractions could be suppressed from native membrane by the presence of  $10\text{mM}$   $\text{Mg}^{2+}$ . However, if  $\text{Mg}^{2+}$  does play a structural role in reaggregated material it must be very tightly bound or deeply buried within the membrane since prolonged dialysis against EDTA failed to cause any disaggregation. It is doubtful if salt linkages are involved in the binding of lipid to protein in either native, reaggregated membrane, or fraction F1 since prolonged dialysis against NaCl solution and washing with water failed to cause any disaggregation. However, the fact that freshly prepared samples of membrane are rapidly precipitated by  $4\text{M}$  salt may suggest that salt links play a more important role under natural conditions. Possibly during preparation of purified membrane significant changes occur in the interaction of membrane components.

In Chapter VI it was reported that solubilisation of fraction F1 could not be detected by P.G.E. after treatment with detergent. However, when dansylated F1 was treated with MAD a marked enhancement of fluorescence intensity occurred. The degree of enhancement was in the same order as that observed when native membrane was solubilised with MAD. This implies that the interaction of detergent with F1 or native membrane results in essentially the same kind of effect upon the membrane protein.

Thus as suggested in Chapter VI, F1 may represent partially reaggregated membrane. Unlike the interaction between F1 and F2 the polar lipids in F1 may recombine without the necessity of  $Mg^{2+}$  as soon as MAD is removed from solubilised membrane by gel-filtration or P.G.E.

Measurements of the polarisation of fluorescence ( $p$ ) of DNS conjugates can give useful information concerning the conformation of protein. Decreases in  $p$  have been correlated with conformational changes involving the unfolding of polypeptides in which all non-covalent interchain forces may have been removed (Horton and Koshland, 1967). Conversely increases of  $p$  may be associated with transformations to more stable states e.g. dimer formation from monomers of F-actin (Tsao, 1953).

In all the membrane-DNS conjugates tested decreases of  $p$  were observed on raising temperature. However, in native membrane  $p$  remained almost constant as temperature was raised until a sudden change at about  $50^{\circ}C$ . This behaviour contrasted markedly with the response of reconstituted membrane and fraction F1 both of which showed a steady decrease of  $p$  as temperature was increased. BSA-DNS tended to show a similar effect with possibly a slight stabilisation up to about  $50^{\circ}C$ . The lipid-rich fraction did not show a response. In this case the protein-DNS conjugate is presumably shielded by lipid, whereas in F1

where there is little lipid present conformational changes of the protein induced by the rise in temperature may be reflected by the drop in  $p$ . In native membrane the interaction of protein and lipid presumably stabilises the protein. Thus it is possible that a major proportion of the protein of native membrane changes physical state at a critical temperature and that under these conditions phospholipids remain more or less unaffected. The fact that reconstituted membrane-DNS responds in a similar manner to F1 suggests that there is very little stabilisation of the reaggregated protein and possibly indicates important differences in the arrangement of lipid and protein between native and reassembled membrane. This may also be reflected in the differences obtained on P.G.E. of MAD solubilised membrane.

Native membrane-DNS was not affected by changes of concentration of NaCl or  $MgCl_2$ . However, decreases of  $p$  were observed when F2-DNS and reconstituted membrane-DNS were treated with increasing concentrations of  $Na^+$  up to  $1M$ . Fraction F1 was not affected by increases of NaCl. Thus salt links sensitive to changes of ionic strength are possibly present in the lipid rich fraction F2 and these same links may also occur in reconstituted membrane but not in native membranes.  $MgCl_2$  did not induce changes in  $p$  of native or reconstituted membrane, but both F1 and F2 showed increases of  $p$  upon the addition of  $Mg^{2+}$ . This implies a stabilisation of protein-DNS moieties in the presence of  $Mg^{2+}$  and may explain the requirements of  $Mg^{2+}$  for reaggregation to occur between F1 and F2.

## Chapter VIII : Discussion

The search for an explanation to the phenomenon of halophilism has stimulated research since the early fifties. Almost all of the cellular components of Halobacterial cells have been shown to be extremely halophilic requiring from 3 to 4M salt for maintaining structural integrity (Larsen, 1967). The basis of the salt requirement has been shown to be ionic rather than osmotic (Onishi and Kushner, 1966).

Baxter (1959) originally suggested that the stabilising effect of salts on halophilic enzymes was due to electrostatic interactions particularly the shielding of negative charges on proteins. Other workers supported this hypothesis and suggested that similar interactions were responsible for the overall stability of the cell envelopes of halophilic organisms, (Brown, 1965; Kushner and Bayley, 1963; Kushner and Onishi, 1966). The theory was supported by the analyses of extracts of halophilic bacteria which revealed an excess of acidic amino acids (Bayley, 1966). However, it has been suggested that electrostatic shielding by salts cannot alone account for the salt requirement of halophilic enzymes (Lanyi and Stevenson, 1970). The salt concentrations which affect halophilic enzymes are in the range 1-4M, whereas electrostatic shielding is thought to be complete at relatively low salt concentrations e.g. 0.1-0.5M. Also shielding cannot

explain differences in the effectiveness of various cations and anions since steric effects are unlikely to be involved with monovalent ions at low protein concentrations (Hochstein and Dalton, 1968; Floss and Hsia, 1967). However, salts do have an effect on hydrophobic bonding in proteins. Nonpolar side chains may form hydrophobic bonds within a protein molecule with one another or with the polypeptide backbone. Sodium chloride and other watersoluble salts which can "salt-out" organic compounds from aqueous solutions tend to stabilise hydrophobic bonds.

The results obtained with ANS upon heating and desalting MDH may reflect changes in two types of bonding; on the one hand interionic shielding and on the other hydrophobic interactions or sidechains. The various changes observed in the experiments described in Chapter III are set out diagrammatically in Figure 8.1.

In the native form hydrophobic bonds may be ruptured by heating to such an extent that the enzyme molecule unfolds even though NaCl is present. However, the presence of  $\text{Na}^+$  prevents total loss of enzymic activity.. But if  $\text{Na}^+$  is removed even at low temperature, the molecule appears to unfold to the same extent and this time activity is zero. Further heating of the native form causes additional decreases in activity as the molecule unfolds and approaches the fully unfolded state.

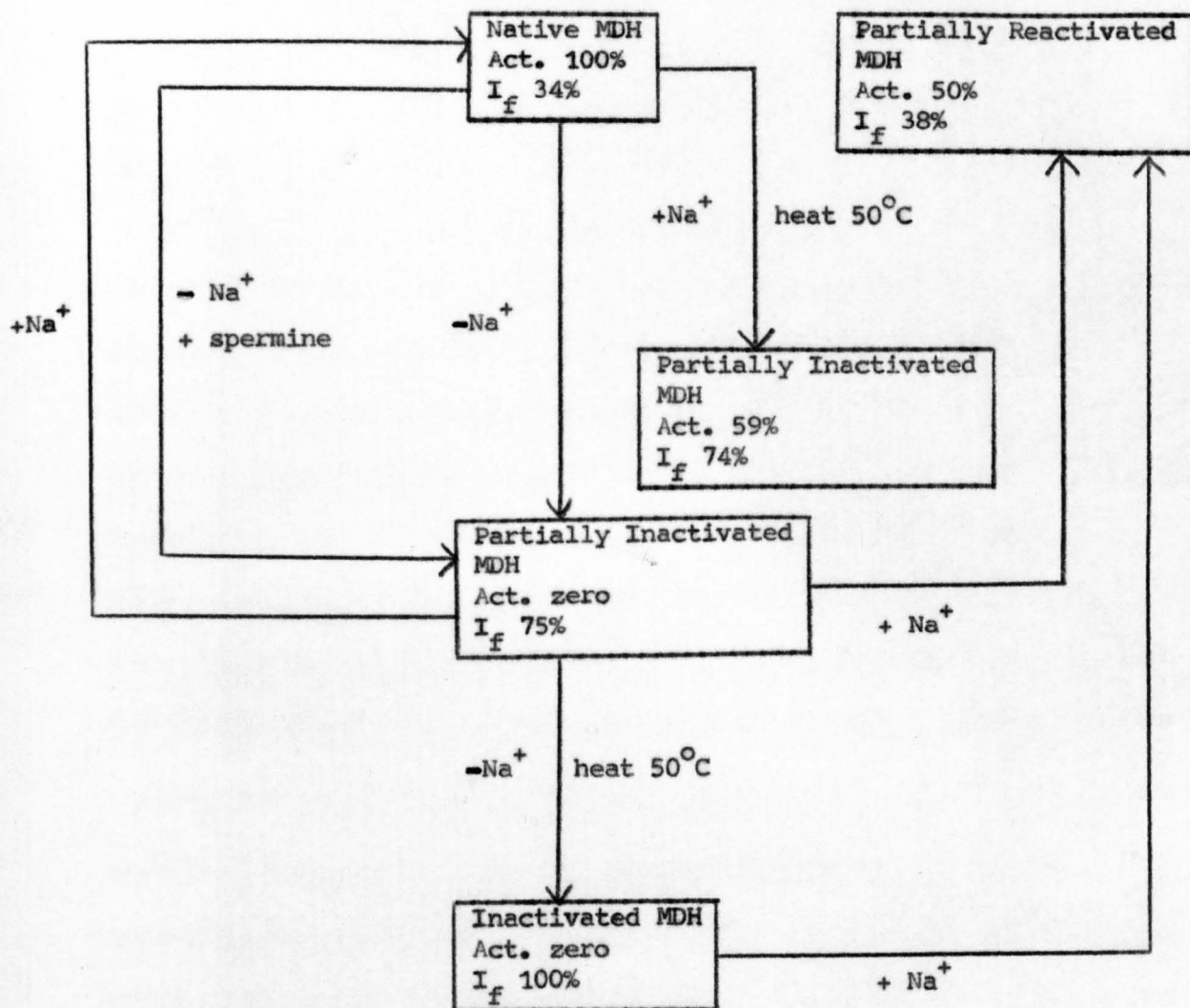


Figure 8.1 Possible relationships of inactive forms of halophilic MDH formed upon removal of  $\text{NaCl}$  or heating as suggested by fluorescence studies with 1,8 ANS and enzymic activities. Activity and fluorescence intensity ( $I_f$ ) are expressed as percent of maximum value obtained.

When  $\text{Na}^+$  is returned to the  $\text{Na}^+$  free form the molecule refolds apparently to the same extent as the native enzyme i.e. the presence of  $\text{Na}^+$  stabilises hydrophobic bonds, but activity is not fully recovered. This may be due to permanent denaturation of an ion sensitive active site. The conformational change due to the interaction of nonpolar sites may be fully reversible since heat denatured salt-free enzyme shows a similar recovery as reactivated salt-free enzyme upon return of NaCl. However a 100% return of activity upon reactivation with NaCl was only obtained when salt had been removed in the presence of low levels of spermine. Spermine is more effective than  $\text{Na}^+$  in counterion effects and possibly stabilises the active site upon removal of NaCl, although zero or negligible activity was detected, presumably because hydrophobic bonds were destabilised upon the removal of salt.

Thus it appears that  $\text{Na}^+$  may be required to activate and stabilise halophilic enzymes by interaction with polar moieties associated with the active site and stabilisation of hydrophobic regions responsible for the overall conformation of the enzyme.

The intermolecular forces involved in halophilic membranes have not been fully investigated. Since the cell envelope of H. salinarium disintegrates on lowering the concentration of salt in the medium very pure preparations of the underlying cell membrane can be obtained. This makes the Halobacteria very good subjects for the study of bacterial cell membranes per se because in other types it



is often very difficult to guarantee that preparations are free of contaminating cell wall material (Salton, 1964). Nevertheless the possibility remains that the nucleotide protein fraction released from crude membrane by gel-filtration on Agarose is of cellular origin rather than membranal. It has not been clearly demonstrated that this material is part of the membrane rather than the cell cytoplasm, but evidence suggests that it represents a rather loosely bound low M.W. highly polar fraction of the membrane stabilised by the presence of  $Mg^{2+}$ . Since the relevance of this component to the membrane was in doubt crude membrane preparations were normally "purified" by gel-filtration in the absence of  $Mg^{2+}$  to obtain so called purified membrane, free of the nucleotide protein fraction. Electronmicrographs revealed that pure membrane from strain 1 and 1M contained abundant unit membrane structures.

The proportion of lipid in this material, about 50% in either strain, is considerably higher than has been found in most other bacteria (see Table 8.1). The fact that "crude" membrane has a lipid content closely similar to membrane preparations of other Gram negative strains further suggests that the nucleotideprotein complex may be considered as an intrinsic part of the membrane,

The structural and fractional significance of the differences found between strain 1 and 1M lipids are not clear, but they

<u>Organism</u>	<u>% lipid in membrane</u>	<u>Reference</u>
Streptobacillus moniliformis	40	Razin and Boschwitz (1968)
E. coli	32	Miura and Mizushima (1968)
Halobacterium halobium	40	Stoekenius and Kunau (1968)
Halobacterium salinarium "crude" membrane	40	Brown (1969)
Pseudomonas aeruginosa	35	Norton et al (1963)
Gram positive bacteria (mean of 16 spp)	24	Salton (1967)

Table 8.1 The proportion of lipid in cell membrane preparations  
of Gram positive and Gram negative bacteria.

must be considered in any theory of membrane organisation. For example, the physical properties of membranes may, in part, be consequences of the packing arrangements of their constituent lipids. In artificial monomolecular films variations in the chain lengths of fatty acids influence the packing of lipid molecules (Van Deenen, 1966; O'Brien, 1967). Thus membrane structure is likely to be affected by the nature of side-chains, degree of branching etc of the component groupings of lipid molecules. Fatty acids of longer chain lengths form much more contracted films than those of shorter chain lengths and unsaturated chains do not pack as tightly as saturated chains of the same length. Branched chains do not pack as closely as unbranched chains because of steric hindrance from the branches (O'Brien, 1967). The major phospholipid component of H. salinarium strain 1 probably contains dihydrophytyl sidechains which being of medium length ( $C_{16}$ ) and fully saturated would tend to favour close packing of lipid molecules. This could have a significant effect on the nature of the membrane especially since a high proportion of the lipid is of this type and cholesterol, saturated lecithins and cardiolipin which tend to have condensing effects on phospholipids are absent, (Van Deenen, 1966; Larsen, 1967). The phospholipid of the colourless mutant, however, does not have the same major constituent as strain 1, and moreover this lipid (spot 4, Figures 5.1 and 5.2) does not incorporate  $\overline{2-}^{14}\underline{C}$  mevalonate possibly implying a markedly different structure from the main component of strain 1 lipid. Unfortunately attempts to elucidate

the structure of this component by mass spectroscopy were unsuccessful. However, the possibility remains that interaction of the lipid components at a molecular level could be responsible for the greater sensitivity of the mutant strain to NaCl levels of the growth medium and strong light. Carotenoid in strain 1 probably protects certain membrane components as has been observed in other organisms (Mathews and Sistrone, 1960; Rottem et al, 1968). Brown (1969) observed a close stoichiometry between menaquinone and carotenoid during the growth cycle. This suggests that carotenoid may protect menaquinone and thus be spatially close to it in the cell membrane. In the colourless mutant a much lower level of menaquinone was found, however, this must be protected to some extent under "normal" light conditions. No carotenoid was found in the mutant, but certain neutral lipids were detected on TLC in a similar position to carotenoid fractions in strain 1. Presumably these lipids fulfil the same function as carotenoid under "normal" conditions.

Recently evidence has been reported that phospholipids tend to exist in a highly fluid state at normal temperatures. McConnell and Huskell (1969) have shown with e.s.r. measurements of spin labelled nerve fibres that small spin label molecules which enter phospholipid layers within the membrane are free to rotate implying that the ligands are effectively a solute in a liquid medium. These workers also found that anaesthetics which are known to act

at membranes increase the fluidity as judged by the behaviour of the spin label. It has been reported that saturated hydrocarbon chains of isolated lecithins are in a highly mobile state at 37°C, (Malhotra, 1970). Thus it is possible that the phospholipids of membranes tend to be in a highly mobile state unless stabilised to some extent by molecular interactions with other membrane components.

Since proteins make up the remainder of the bulk of the membrane they are the most likely candidates for interaction with the lipids. However, a major proportion of the protein found in a particular membrane may not be directly involved in the basic membrane structure. Some enzymes may belong to this category. Recent biochemical investigations of the membrane of human erythrocyte lipid free ghosts have led to the suggestion that there are several distinct species of protein present in the membrane matrix (Rosenberg and Guidotti, 1968; Blumenthal, 1968; Marcheri and Steers, 1968). Other workers investigating more complex membranous systems, e.g. mitochondria (Criddle et al, 1962; Richardson et al, 1963; Woodward and Munkres, 1967; Lenaz et al, 1968) and chloroplasts (Criddle and Park, 1964) have reported the presence of membrane proteins which are insoluble in water at neutral pH and bind phospholipid hydrophobically. No enzyme activities have been detected in the preparations from mitochondria and chloroplasts and since they represent up to 50% of the membrane protein a



structural role has been proposed (Criddle, 1969). Non-catalytic proteins are however difficult to identify and purify since no specific assay is available. In spite of this difficulty recent attempts have been made to obtain structural proteins from bacteria by extractions based on the methods used with mitochondrial membranes. The proteins obtained have in general character been strikingly similar to the structural proteins obtained from other membrane sources (Mirsky, 1969; Brown and Pearce, 1969; Schnaitman, 1970).

The lipid-free membrane protein preparations of H. salinarium were particularly hydrophobic in character having similar proportions of polar and apolar amino acids as SP isolated from several other sources. The SP preparations obtained from strain 1 and 1M by traditional methods constituted about 20% of the membrane protein and were even more apolar than typical mitochondrial or bacterial SP prepared by similar procedures (Table 5.5). The ratio of hydrophobic to apolar amino acids in these samples approached values obtained with myelin proteolipid. It is interesting to note that like Halobacterial membrane myelin has a very high lipid content although of a somewhat different composition (Malhotra, 1970).

A feature of Halobacterial lipid is the particularly high phospholipid content, conferring a strong overall polar character

to the lipid. Evidence however, suggests that the main interaction between the protein and lipid of the membrane is hydrophobic. Strongest indication of this is the finding that over 75% of the lipid can be removed by the action of anionic detergents which disrupt hydrophobic bonds. Also the addition of MAD to dansylated membrane caused a substantial increase of fluorescence intensity indicating a major conformational change of the membrane protein. Urea which will disrupt ionic or hydrogen bonds causes the release of about 35% of the membrane protein but only trace amounts of lipid were detected in these fractions. The protein released by urea was of a very polar character while the residue was extremely apolar. Similarly the protein-rich fraction (F1) isolated after detergent treatment was also highly apolar and represented 80% of the membrane protein. The major fractionations of crude membrane are shown diagrammatically in Figure 8.2. It seems that the bulk of the protein is highly apolar and reacts with lipid via hydrophobic interactions. It is unlikely that the protein-rich fraction (F1) represents a subunit of the membrane since on P.G.E. it was shown to be composed of several distinct protein species. Probably on removal of MAD by gel-filtration substantial reaggregation occurs even in the absence of  $Mg^{2+}$ , to form the particulate structures which are clearly visible in ems.

Complete reaggregation of the protein-rich and lipid-rich fractions requires the presence of  $Mg^{2+}$  or spermine. Fluorescence

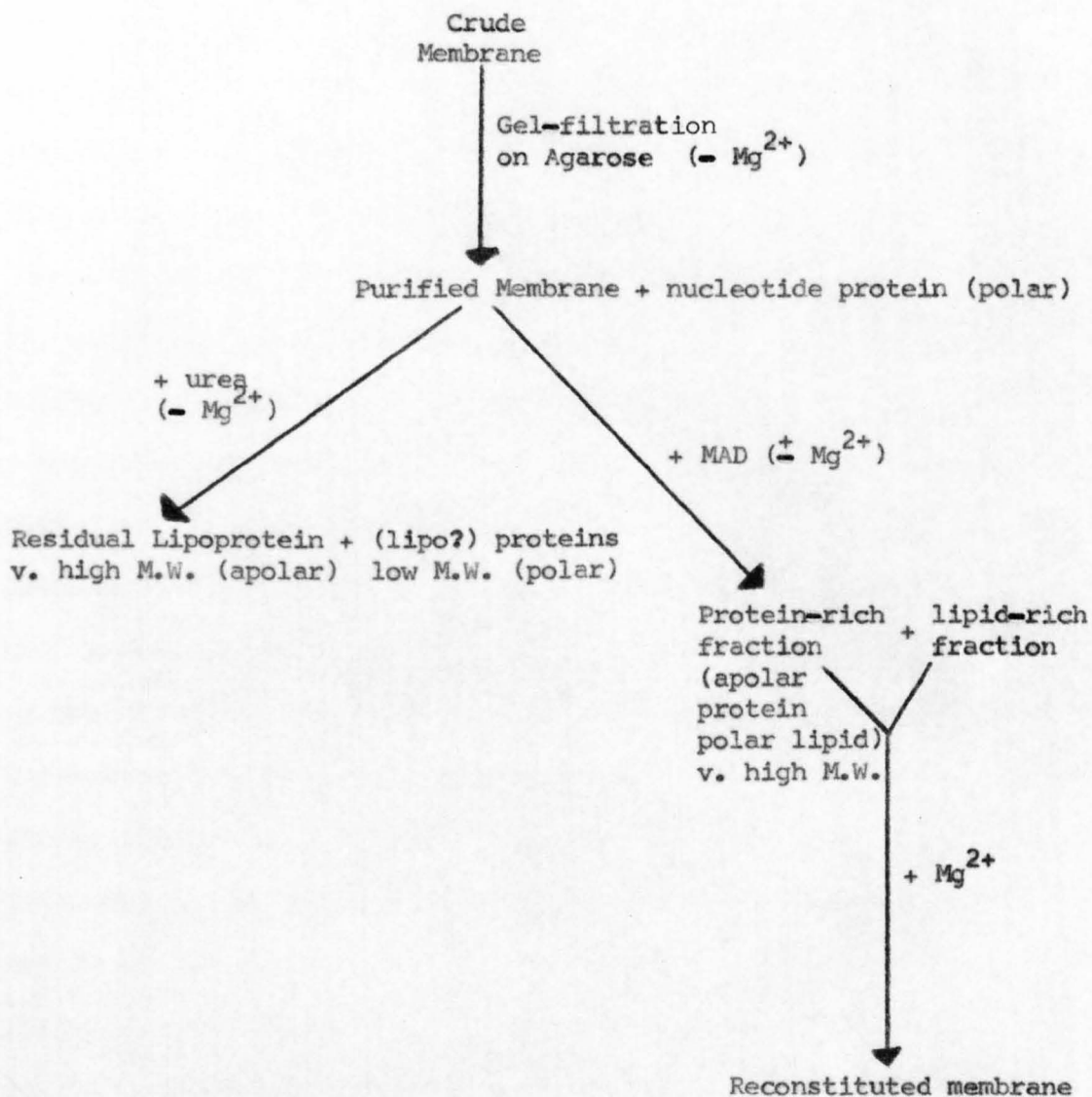


Figure 8.2 The fractionation of the cell membrane of H. salinarium



experiments in which these fractions are labelled with DNS indicate that both are stabilised by the presence of  $Mg^{2+}$  whereas no similar effect was detectable with native or reconstituted membrane. The release of polar proteins from membrane by urea or gel-filtration can also be suppressed or markedly reduced by the presence of  $Mg^{2+}$ . Thus possibly  $Mg^{2+}$  is required to neutralise negative groups on the polar moieties or form salt links to promote binding. However, F1 and F2 are basically apolar proteins although the lipids must contain substantial numbers of polar phosphate groups which might require stabilisation with  $Mg^{2+}$ . Alternatively detergent may be bound via hydrophobic links to the protein or lipid and the detergent anions may require neutralisation before aggregation will occur. Gel-filtration of detergent alone indicates that most of the MAD can be recovered in separate peaks and for the reaggregation experiments F1 and F2 were purified by dialysis. Rottem et al (1968b) showed that 99.99% SDS could be removed from solubilised Mycoplasma membranes by gel-filtration on Sephadex G25. Thus presumably F1 and F2 are not contaminated with detergent. Polarisation of fluorescence of dansylated reconstituted membranes was, like native membrane, not susceptible to changes of  $Mg^{2+}$  concentration. Thus interaction of F1 and F2 probably results in a stabilisation of the sites at which  $Mg^{2+}$  reacts. If  $Mg^{2+}$  plays an important structural role in the reformed membrane, it must be deeply buried or tightly bound since dialysis against EDTA failed to cause

disruption of reconstituted membrane. This could only be achieved with detergents suggesting that as in native membrane hydrophobic interactions were of prime importance in maintaining the integrity of the membrane.

However, although reconstituted membrane also resembled native membrane in appearance in so far as triple layered structures, and vesicles were visible, it was apparent from P.G.E. of detergent solubilised membrane and fluorescence experiments that significant differences existed between native and reaggregated membranes. This difference was particularly noticeable in the responses of dansylated material to temperature changes.

Polarisation of fluorescence measurements of heated DNS-F1 indicated a steady destabilisation as temperature increased. This was in contrast to native membrane which showed little change up to about 50°C and was then destabilised rapidly. Glazer et al (1970) measuring circular dichroism of erythrocyte membranes found that a substantial proportion of the membrane proteins changed from  $\alpha$ -helix to random coil configuration as temperature rose from 25°C to 80°C without any apparent effect on the phospholipids of the membrane. Similarly in H. salinarium dansylated fraction F2 showed very little response to temperature changes. However, on reaggregation the resulting reconstituted membrane behaved like F1 without any apparent stabilisation to temperature change.

Thus the protein moiety of the membrane appears to be destabilised to heat and susceptible to changes of  $Mg^{2+}$  concentration upon removal of lipid. The lipid fraction, on the other hand, is more or less unaffected by heat increases but susceptible to destabilisation with high levels of  $Na^+$  as well as stabilisation with  $Mg^{2+}$ . Reconstituted membrane was also destabilised with  $Na^+$ . This may imply that salt links are of significance in reconstituted membrane, but not in native membrane which is unaffected by increases of NaCl concentration. Thus attempts to obtain stabilisation of F1 and F2 by reaggregation thereby seeking to demonstrate the importance of hydrophobic protein-lipid interactions in maintaining the integrity of the membrane were not wholly successful. Other workers have also found significant differences between native and reaggregated membranes (Butler et al, 1967; Tillack et al, 1970). The latter have provided electronmicrographs of freeze-etched reformed Mycoplasma membranes which reveal differences in the organisation of the membrane matrix. Perhaps these results are not surprising since it has been shown that considerable conformational changes occur when proteins are treated with detergents such as SDS, (Tanford, 1968) and these may not be reversible. Nevertheless the results of Tillack et al indicated that the reformed membranes of Mycoplasma possessed certain features which resembled myelin or artificial phospholipid membrane bilayers. Thus the possibility arises that native membranes may not universally contain a lipid bilayer structure as suggested by Danielli and

Davson (1935) and Robertson (1959) but possibly an alternative configuration of protein and lipid along the lines of contemporary theories (Branton, 1969; Korn, 1969b) in which protein-protein and lipid-protein hydrophobic interactions are the basic forces holding membranes together with polar groups of both proteins and lipids orientated at aqueous interfaces, (Green and Perdue, 1966; Green, 1968; Lenard and Singer, 1966; Vanderkooi and Green, 1970).

The maintenance of the integrity of the basic cell membrane of H. salinarium does not require the presence of high levels of  $\text{Na}^+$ . However, the membrane bound enzymes are in general particularly halophilic and resistant to reactivation after removal of salt. As mentioned above the halophilic proteins are often highly polar and possibly the polar moieties which were released from the membrane by urea or gel-filtration in the absence of  $\text{Mg}^{2+}$  represent halophilic proteins which in vivo are intimately connected with the enzymic activity of the membrane and exposed to the high extra and intracellular salt levels. It has been shown in non-halophilic organisms that some functionally defined proteins, such as cytochrome c and ATPase in Streptococcus faecalis, are bound tightly to the cell membrane apparently externally to the lipid (Chapman and Wallach, 1968). The interaction between these proteins and the lipid is thought to be interionic for cytochrome c and via a  $\text{Mg}^{2+}$  complex for the bacterial ATPase. Thus it is possible that the high M.W.  $\text{Mg}^{2+}$  stabilised fraction released

by urea from H. salinarium could represent such a complex as ATPase. Recently Lanyi (1971) has shown that cytochrome oxidase is released from the cell membrane of H. cutirubrum at low salt concentrations and has suggested that the binding responsible is essentially ionic in character. Cytochrome oxidase is one of the few enzymes of membrane origin which can be successfully reactivated from the salt-free form (Larson, 1967). The fact that other membrane bound enzymes cannot be reactivated after removal of salt during the isolation of the cell membrane constitutes a major disadvantage in the use of Halobacteria for the study of the bacterial membrane but may reflect the intimate structural relationship existing between the enzymes and the cell membrane.



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